

Self-reliance means self-denial

The Indian government seems to be changing its policy on the import of technology. Far from undermining Indian hopes, this may be the best assurance that they will come true.

To what extent should developing countries seek to keep up with the industrialized countries of the world in technology? And how should they attempt to do so? Unhappily, there are many places in the world where the question is strictly academic. For most of Africa south of the Sahara, for example, the notion that indigenous science and technology might contribute to the solution of immediate problems is, for the time being, an impossible dream (which is not to say that it should be forgotten). But there are other states, of which India is the chief example, where governments have for decades sought to drag themselves into the future by devoting resources to research and development. Often, as a consequence, they have run into criticism from those elsewhere who complain that when resources are so scarce, they should be spent instead on more immediate tasks, the relief of poverty perhaps. That argument is mistaken (and patronizing as well), but that does not absolve developing countries from working out sensible policies on research and development.

The need is illustrated by the public row in India during the past month over the new government's policy on semiconductors. At budget-time last month (see *Nature* 4 April, p.496), the Indian government announced several measures to liberalize the import of technological equipment from overseas — computers, for example, and also announced an arrangement with the Hemlock Semiconductor Corporation of the United States under which the overseas company will build and license a polysilicon plant in India. This decision has aroused opposition within India from three sources — those parts of the Indian research establishment that complain that the deal demeans their work, industrial companies that consider the arrangement an unfair challenge which robs them of opportunity and those who argue more generally that dependence on overseas technology is inappropriate for a country such as India. The government's resolution of the argument, announced last week, is a compromise: the arrangement with Hemlock will go ahead, but the Mettur company, which has built a 2-tonne a year pilot plant for manufacturing polysilicon by a process developed at the Indian Institute of Science at Bangalore, is also to be encouraged to build a 25-tonne plant at Baroda. Meanwhile, according to the electronics minister, Mr Shivraj Patil, the indigenous technology will be further encouraged by the placing of research contracts with laboratories such as the National Chemical Laboratory at Puna. By what tests does it make sense to follow both paths, keeping a dog but being prepared to bark oneself, so to speak?

Self-reliance

The answer, convincing enough, is complicated, and turns on the distinction commonly made in India between self-reliance and self-sufficiency. Self-reliance is that state of grace in which a country embodies the technological skill needed to accomplish all known goals, whence the considerable Indian investment in space research. Self-sufficiency is more demanding, requiring not merely the skill but the capacity (technical and financial) to do everything. Since independence in 1947, Indian governments have been wedded to the doctrine of self-reliance, and have also flirted from time to time with that of self-sufficiency (whence, in part, the cumbersome system of import controls now being dismantled).

The doctrine of self-reliance is especially confused in its

application. India's need, like that of other developing countries, is for a degree of technical competence that will suffice to meet the unknown demands of the future. One ingredient of any provision to this end must be institutions at which scientific work (teaching as well as research) of the highest quality is carried on. India is reasonably but not lavishly provided in this respect: there is a handful of fundamental research establishments of international standard, but the quality of training and research within the university system is too patchy to ensure that the fullest use is made of talent. More serious questions arise in the application of technology. Since the 1950s, India has maintained its large nuclear energy establishment for two declared reasons — to provide a model of the indigenous application of technology in a daunting field, and to maintain independent control of an industry whose importance ranks high, politically and economically. In the first respect, the policy has worked well: the Indian Atomic Energy Commission is an important source of technical expertise. But India would have had more rapid access to nuclear power if it had been able to sign contracts for building power stations with overseas suppliers and to put up with controls on the use made of plutonium. This is the lesson that Mr Rajiv Gandhi now seems to have applied in the smaller matter of the semiconductor industry.

Prudence

In prudence, he should now go a step further. A huge half-developed half-developing country such as India has two needs of technology — the chance to manufacture products that compete on world markets, thus earning the foreign exchange needed to pay for imports, and the promise that, in the future, there will be even more sophisticated things to make and sell, domestically and overseas. Traditionally, Indian governments have sacrificed the first need to the second. The notion that India might successfully build an industrial base by licensing technology from overseas, and only afterwards embellish that with its own distinctive contributions, has often given great offence. Yet this is how many other modern states have prospered. In India, a further benefit of a policy typified by the Hemlock deal would probably be that Indian technology would be improved in those respects in which it is at present weakest, the management of projects. Mr Gandhi's government should build on the precedent it has now established, looking for opportunities to benefit from technology now, not later. Far from being a threat to what may lie distantly ahead, a policy of judicious exploitation of other people's technology is a necessary foundation for the launching of more ambitious enterprises in the future.

The bearing of the Indian problem on the condition of other developing countries is remote, but not entirely negligible. While the world wrings its hands over the famines sweeping much of Africa, it tends to be forgotten that the agricultural deficits that afflict them have been outlawed in India by the intelligent application of technology, that of agriculture. Experience not just in India but in the United States has shown that the improvement of food production requires not merely technical innovation (new seeds especially) but means by which farmers, often peasant farmers, can be helped to use them efficiently. Yet during the past twenty years, this combination of research and extension service has been eroded in many of the countries of West Africa



where it was once productive. Many other developing countries have never benefited in this way. The moral for them is that priority should be given to the development of agricultural research. The other leaf that should be taken from the Indian book is the demonstration of competence in the pursuit of science, if only so that too much talent is not lost too soon. □

Powell bites dust

The British government needs to think hard now about surrogacy.

THE British government has done the decent (and courageous) thing by deciding that it will not lift a finger to save Mr Enoch Powell's bill on *in vitro* fertilization from being lost in the procedural hazards of the House of Commons (see p. 573). Common sense requires that there should be an opportunity to consider carefully how research in this field should be regulated. That regulation of some kind is necessary is agreed by everybody, even those who work in laboratories, but there are serious doubts whether the 14-day limit suggested by the Warnock committee is either appropriate or workable. It is to be hoped that the government will now consider seriously whether regulation by public ethical committee would be preferable, not least as a way of meeting the public concern that research with living human embryos should be responsibly designed and soberly directed. That course will not comfort those (Mr Powell included) who hold that the whole idea is an abomination. That is the sense in which the government has shown courage — and is the reason why prudence again requires that it should press ahead with its own legislation as quickly as it can.

To take the edge off the complaints there will be when the Powell bill is defeated next month, the government plans that there will be an outright ban on what is called commercial surrogacy, the practice by which healthy women offer the service of gestation to others for a fee. Warnock would have banned all surrogacy, but an important minority opinion on the committee held that some allowance should be made for exceptional circumstances. The plain truth, now, is that nobody knows how common may be the practice in countries such as Britain, and there is only guesswork to suggest what may be the circumstances (apart from the infertility of the intended mother). Yet reports abound of women providing this service for infertile sisters, while there is no way of knowing what small proportion of illegitimate births followed by adoption are essentially surrogacy arrangements.

That is one reason why the outright ban on "commercial" surrogacy now proposed deserves more careful consideration. In the spirit of the present time, which rightly allows an adopted child to learn something about its natural parents at majority, frankness about genetic origins is in fashion (and could, sometimes, be medically important in tracking genetic defects). Should these principles not also apply to children born of surrogacy in the forms now accepted? Will such practices, tacitly condoned at present, become crimes if the surrogate mother is rewarded in kind, not cash? And what is to happen if the small but unavoidable risks of pregnancy to the mother should become realities? Do her dependants go neglected? These questions may be hypothetical, but they are also important.

The British government has started from the other end of the problem — the publicity and the attendant fuss following a surrogate birth last year in which a commercial agency had been involved. Would the fuss have been as great if the agency had been a registered charity, organized perhaps as if it were an adoption agency? Almost certainly not. This is but one reason why the British government should be prepared to allow for exceptions to the rule it is now planning to introduce. The guiding principles should be that surrogacy should not be allowed to make anybody's fortune but that women who volunteer to provide the service should be safeguarded and repaid for their time and trouble, preferably in a manner open to public scrutiny. Is that too much to ask? □

International manners

The US administration should not offend its friends overseas by tactlessness.

NEXT month's meeting of the heads of government of the major industrialized nations, to be held at Bonn in West Germany, has all the makings of a rough-house. On this occasion, the hard core of the agenda, economics, will be the chief reason why the United States will find itself pinned uncomfortably in a corner. But US policy on strategic arms is likely also to sour what people have to say to President Ronald Reagan. Continuing doubt over US intentions over its star wars plans is one bone of contention. Italian President Bettino Craxi was right to complain last week that the US administration owed Mr Mikhail Gorbachev a less ungracious reply to the Soviet offer to suspend the deployment of SS20 missiles aimed at Europe than the jaundiced comment that it was a mere propaganda ploy.

The substance of these issues has been much debated, here and elsewhere, but the manner in which the United States now conducts its international business is becoming an obstacle to its attainment of its objectives. Even those who agree with what the administration is trying to achieve must be dismayed. Mr Gorbachev, after all, declared that the Soviet Union would unilaterally halt the deployment of SS20 missiles within range of Western Europe until November, and would continue a moratorium thereafter if mutual agreement was reached by then. The proposal is full of snags, while the plan to make it public at this stage is a breach of the agreement between the superpowers to restrict their negotiations on strategic arms to the closed sessions at Geneva. But even a thoroughly sceptical administration could have contented itself with something like "High time!" or "There'll be time to talk about that at Geneva". Last week's needlessly hard lines will have made many third parties ask who are the bad guys now.

Much the same is true of the US administration's public statements on the international money problem. In the past few days, US cabinet officers have too quickly taken up President Reagan's cry last month that there would be no imbalance in the world economy if only European states were as productive as the United States, which is at best a half-truth and which may even be more false than true. (If Europe were as productive as it might be, the United States would not be able to fund its present deficit by borrowing from abroad except by pushing interest rates still higher.) The irony in this is that the United States seems not to have been restrained in its attempts to correct its trade balance with the most productive of its international competitors, Japan. But the proposal now being canvassed by officials in Washington that would-be US exporters should be represented on the regulatory committees that will deregulate the supply of equipment to the Japanese telephone system will seem especially demeaning in Japan, where it will be recognized that the proposal would be constitutionally impossible in many places, and in Europe, where it will be asked why only US exporters should enjoy this privilege.

Complaints such as these that the United States too often behaves as if its own interests are paramount are not new. Most people also acknowledge that the United States has a special role in world affairs, militarily and economically. The sympathetic among them also appreciate the difficulty of a government such as that of the United States, with huge domestic preoccupations and watched over by a necessarily parochial Congress, in finding forms of words to explain itself that do not raise hackles elsewhere. Yet the administration's performance seems to be getting worse.

Part of the trouble may be the number of new faces, or of old faces in new jobs, that have made their appearance at the White House and in US government agencies since the beginning of the year. That is the charitable explanation, in which case Bonn may be a stimulus for changing tack and for learning that international relations are as easily soured as personal relations by tactlessness. Let us hope the explanation is not more sinister. □

Research secrecy

Pentagon plays havoc at optical conference

Washington

THE US Department of Defense (DoD) caused severe disruption at a technical symposium of the Society of Photo-Optical Instrumentation Engineers (SPIE) in Virginia last week when it withdrew at a few days' notice some 50 papers previously scheduled for presentation. The titles of the papers and some of the abstracts had been cleared for publication by DoD, but many of the texts were later found to contain either classified information or information subject to export regulations.

Eventually, most of the papers were presented (after some censorship) in a new type of "export-controlled" session, attendance at which required US nationals to certify that they would keep technical information confidential and which required foreign nationals to be sponsored by their embassies. Twelve of the 50 papers were declared classified and were not presented.

DoD last year declared that it would rely only on classification to restrict publication of fundamental research carried out on university campuses. As universities do not generally carry out classified research on campus, this seemed like a guarantee not to interfere with academic work. But the papers restricted at the SPIE conference were based on research by private contractors receiving DoD funds in category 6.3, which covers research that is potentially militarily sensitive and classifiable. Research in category 6.3 is not generally considered to be "fundamental", but none of that affected at the SPIE conference had actually been classified. Private research contractors, in contrast to universities, do accept contract clauses requiring researchers to submit papers to DoD for pre-publication scrutiny — effectively a licence for DoD to prevent dissemination of the information should it so choose.

DoD says that all of the 50 affected papers would have had to be suppressed totally, by invoking prepublication review clauses, but for a new authority in the 1984 Defense Authorisation Act, under which DoD could allow most of the papers to be given, but on condition that those present would agree to the confidentiality requirements of export-controlled sessions.

Conference organizers say that no foreign national wanting to attend one of the two export-controlled sessions was denied access, but a number of them at the conference decided, for various reasons, not to seek admission to the controlled sessions, which dealt with synthetic aperture optics and wavefront sensors. Both areas have applications to astronomy as well as to weapons design: adaptive optics may be

used to "untwinkle" stars in large telescopes, while synthetic aperture interferometry may allow many optical telescopes to be combined.

In the 1984 Defense Authorisation Act, DoD was given new authority to deny public access to unclassified technical data in its control that are judged to contain critical technology with military or space applications and which cannot be exported without restriction under Export Administrations Regulations. Previously, the department had no authority to withhold such technical information when faced with a request under the Freedom of Information Act.

It is now DoD policy to oppose the open distribution at conferences of unclassified technical data subject to export control. But DoD is also authorized to make such data available to those who are prepared to guarantee its confidentiality, which mechanism DoD brought into play for the first time at the SPIE conference. The exemption was granted by Congress in response to the concern of small defence contractors, which feared that they would otherwise be denied data necessary for

them to bid competitively for DoD contracts.

The American Civil Liberties Union and the American Association for the Advancement of Science expressed concern last week that DoD had used a legal authority applying to DoD-controlled data to restrict public access to research material still the property of the contractors. DoD's explanation is that it still relies on contractual restraints to prevent open publication, and that only the exemption of those guaranteeing confidentiality was derived from the 1984 Authorisation Act. But, paradoxically, the exemption mechanism that DoD brought into play last week could in practice allow DoD to impose tighter control through contract mechanisms. Many allied countries fear that the United States is tempted to restrict foreign access to cutting-edge technology because of its commercial rather than its military importance.

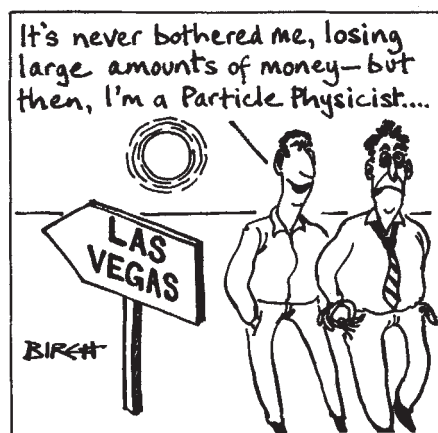
Much of the very visible annoyance at last week's conference was due to the lateness of DoD's intervention. Sessions were being rearranged from one day to the next. But DoD says this was a practice run at making some export-controlled data available in unclassified but restricted sessions, and says that the lateness was due to the failure of another scheme which had been proposed — to have two entirely classified sessions running in parallel with the regular conference. Only one classified session was in fact held. **Tim Beardsley**

Accelerator scrutiny in prospect

Washington

THE US House of Representatives Science and Technology Committee has warned that it intends to treat particle physicists sceptically when considering construction of the proposed Superconducting Super Collider (SSC).

In approving the administration's



request of \$20 million for continued research and development on the accelerator project, much less than the \$60 million that the Department of Energy (DoE) had asked for, the committee said that it doubted whether DoE's goal of starting construction by 1988 could be met.

Still smarting over its experience with ISABELLE, Brookhaven National Laboratory's \$200 million fiasco, the committee said it would not even consider a proposal for SSC until DoE could prove that the superconducting magnets will function as planned for the advertised price. ISABELLE, delayed for years, was abandoned in 1983 after unexpected problems in design of the superconducting magnets. Although the problems were eventually ironed out, events in physics had in the meanwhile made ISABELLE obsolete, but not before a 2¼-mile-long tunnel, control room, experimental areas and magnet production facilities had been built.

Optimistic projections put the cost of the SSC magnets at 15 per cent less per unit of length than those now operating at Fermilab; at \$5,000 per metre and assuming an 80-km ring, that works out at \$400 million for the magnets alone. The current estimate for the total cost of SSC is still \$3,000 million, not including detectors, research and development and allowance for inflation.

Leon Lederman, director of Fermilab, said that previous proposals had been approved by congressional committees almost "by return mail". "We're bearing the cross of ISABELLE", he said.

Stephen Budiansky

French geoscience

New umbrella wins friends

FRENCH oceanographers, geophysicists, geologists, astronomers and space scientists have clubbed together to form a new organization, INSU, the Institut National des Sciences de l'Univers, which will manage big research programmes under the financial guidance of the principal French research council for basic science, CNRS (Centre National de la Recherche Scientifique). INSU should give academic oceanographers, at least, more weight than hitherto in French science politics.

The institute has been created only after a fight from geophysicists. INSU is based on its predecessor INAG (l'Institut National d'Astronomie et de Géophysique), where the small but effective French community of geophysicists played a strong role. INSU, to their chagrin, includes the more traditional field geologists who outnumber the geophysicists more than ten to one. Some geophysicists, who see themselves as the vanguard of French geoscience, have strongly resisted the move. The struggle continues in the universities and in the laboratories of CNRS, where geophysicists argue that geologists should relinquish more of their posts to the "new geology".

Within INSU (which will be directed by meteorologist André Berroir of the Ecole Polytechnique), realism prevails, and the talk is of new big programmes that will unite the warring tribes along the lines of the recent survey of Tibetan geology and geodynamics headed by Claude Allègre of the Institut de la Physique du Globe. Possible programmes for 1986 include a study of Caribbean tectonics and work in Latin America on the Cordillera.

Geologists themselves will also benefit from the creation in INSU of a working group on medium-scale computers for the earth sciences. Undersupplied with computers, as are many French scientists, geologists expect to need powerful mini-computers to process image data from SPOT-1, a new-generation French Earth observation satellite to be launched later this year. Both science minister Hubert Curien and Berroir, the INSU director, are in favour, even if that means using foreign machines. (Berroir masterminded the first French purchase of a non-military Cray-1.)

Oceanographers feel that the institute will strengthen academic science. In France, the field has been long dominated by the technological interests of CNEXO (recently transformed into IFREMER, the Institut Français pour l'Exploitation de la Mer, by the addition of fisheries research.) INSU should provide oceanographers with some political power, authority and even money with which to strengthen the pursuit of basic science in the face of IFREMER's largely applied bias. Hitherto, they have had to rely on a small understaffed interdisciplinary programme of the CNRS, the

"PIROCEAN" programme.

PIROCEAN, with its FF 10 million (£1 million) budget, will now be absorbed into INSU, where it will add to the FF 35 million (£3.5 million) brought by INAG and undoubtedly gain greater influence than it had when directly under the CNRS umbrella.

The new merger should also comfort CNEXO oceanographers whose 450 technical research staff were in danger of being swamped by the 600-700 scientists of the fisheries research organization in the earlier IFREMER merger. (That union also squeezed the research budget of both organizations, as salaries of the old fisheries organization were raised to match CNEXO's without a corresponding in-

crease in total funds.)

Astronomers, meanwhile, who looked to INAG to support observatories and new telescopes, remain blissfully unconcerned. (French membership of the European Southern Observatory is paid for by the foreign ministry.) Plans are afoot for THEMIS, a high spatial resolution polarizing solar telescope for Tenerife (in collaboration with the British, Sweden, Denmark, Spain and West Germany) and for the completion of the millimetre-wave interferometer IRAM in the south of France (due to operate as an interferometer some time in 1987). But this year will be the first for which the astronomers (and geophysicists) have had to fight for large programme funds with geologists and oceanographers. The financial effects of the INSU merger will not be clear until the budget for 1986, due this autumn.

Robert Walgate

UK biotechnology

Public lab. makes private deal

ONE of the most unusual arrangements yet for turning public investment in research into industrial channels, Porton International, announced last week an arrangement with the British government that gives it the right of first refusal on biotechnology innovations at the Centre for Applied Microbiology and Research (CAMR), the laboratory at Porton Down in Wiltshire where British research in biological warfare was once carried out, but which was transferred to the Public Health Laboratory Service seven years ago. The same stretch of Wiltshire down remains the site of the Chemical Defence Research Establishment, operated by the Ministry of Defence.

The new arrangement is the outcome of several years of negotiation, most recently a process in which other major British companies had been asked to express an interest. Porton International already has agreements with CAMR for the exploitation of two of its proprietary developments, asparaginase (used in cancer chemotherapy) and a vaccine for herpes simplex virus (based on developments at the University of Birmingham as well as at CAMR).

In a joint statement, the company and the Public Health Laboratory Services Board said that the new arrangement will run for thirteen years from 1 April, and that the arrangement might be continued thereafter if neither party saw fit to pull out. A spokesman for Porton International said that developments not taken up by the company would remain the property of CAMR, and might be offered to other companies. But he suggested that such happenings would be few and far between.

The management of the laboratory will remain the independent responsibility of the director, Dr Peter Sutton, the Public Health Laboratory Service and ultimately the Department of Health and Social Ser-

vices. But the existence of the new agreement is likely to divert the scientific interests of many members of the laboratory into directions that may be profitably exploited.

The agreement covers not merely biological materials produced by innovative methods but also new processes that may emerge (as for the manufacturer of phenylalanine and the intra-conversion of steroids). Apart from the materials already being brought to the market, there are plans for the production of frozen cell pastes (for the derivation of restriction enzymes), the production of enzymes (such as carboxypeptidases), vaccines other than herpes simplex (such as against pertussis virus) and cloned human hormones (such as human thyroid and follicle-stimulating hormones).

Under arrangements established before 1 April, Porton International is already financing the work of several members of CAMR. Nobody is as yet prepared to guess how large a proportion of the total cost of running the laboratory will derive from royalties provided by the company on sales of proprietary products. But a spokesman for the company guessed that more than half the 250 members of staff at CAMR were working on projects with some commercial interest, but that the laboratory's basic research was also essential to the success of the new arrangement.

Porton International Ltd is for the time being a holding company, with a number of subsidiaries (such as LH Fermentation) active in fields related to biotechnology. The recent development appears to have been financed by capital contributions from a number of financial institutions, including the pensions funds of several public companies and a number of insurance companies. The chairman of Porton International is Wensley Haydon-Baillie. □

Israeli science

Optimism from new minister

Rehovot

ISRAEL'S new science minister, Mr Gideon Patt, has a great many plans but very little money with which to carry them out. Nevertheless, he remains optimistic.

"As minister of commerce and industry", he declares, "I helped promote applied research. Now, as minister of science, I hope to boost basic research. And just as I initiated a five-year plan for industrial development in my old post, I now intend to initiate such a plan for the development of science and technology".

Patt points out that Israeli investment in science and technology now stands at 2.2 per cent of the country's gross national product (GNP), which is, as he puts it, "a respectable figure", and more than is invested, for example, by the Netherlands. But he hastens to add that the percentage figure is misleading on two counts. First of all, Israel's whole GNP is not large and so the total annual investment in research and development is around US\$700 million, less, that is, than is spent on research and development by some individual US companies. Moreover, more than half of the available funds go to military research and development, from which the economy benefits to only a limited extent.

In spite of the problems in regard to applied research, Patt is far more concerned with the decline in funds available for basic research. He notes that the universities, where almost all basic research takes place, once allocated some 50 per cent of the funds for education and the other 50 per cent for research. In the past five years, however, because of massive cuts in government support, they have greatly reduced the money they allocate for research.

"Thanks to superhuman efforts by our scientists", Patt says, "we have not so far fallen behind. However, sheer devotion and ingenuity cannot make up for the lack of funds indefinitely and if something isn't done soon to remedy the situation, Israel will become a scientific backwater, just another little country with enormous problems and few resources with which to solve them."

Patt's solution, one already on the stocks, is to create a special \$250 million fund from overseas sources for scientific research in Israel. This will yield up to \$30 million a year to support research, a sum that will be supplemented by another \$10 million from Israeli government and binational sources.

Why is Patt, a non-scientist, interested in basic research? "I'm not", he says, "I simply realize that without basic research, there will be no innovative applied research, no original products. And if we just base our industry on secondhand research, we will never be able to keep up with our competitors."

The science minister is certain that proper support for research will pay off, generating enough income, through new products, to repay investors in his special fund and to strengthen the economy.

Ultimately Patt would like to see his ministry of science turned into a ministry of science and technology. This would, he believes, facilitate the kind of overall planning that does not exist at present. More specifically, the chief scientists now found in most ministries, where they are charged with helping to find science-based solutions to ministerial problems, would be linked directly to the ministry of science and technology and would be guided by the overall goals that it sets.

In the meantime, Patt has already taken a first step towards greater coordination by creating a regular forum where all the chief scientists meet under his chairmanship at regular intervals. He is now planning to expand the scope of this forum by inviting

university vice-presidents for research and development to participate.

Patt has also turned his attention to the shortage of trained engineers for Israeli industry, which already needs 2,000 more than it can find. He supports attempts to bring back some of those now in the United States, though he sees this, at best, as a partial solution. He has therefore joined hands with the army and with the manufacturers' association to establish a special school where practical engineers with two years of post-secondary training could be turned into qualified engineers in two years. But this school may never be created because the universities have tentatively reversed their long-standing opposition to such a scheme and will probably inaugurate a similar programme themselves.

But the new science minister sees real significance in his present position. "Ten years ago", Patt declares, "the ministry of science wasn't very important. Today it is already important. Ten years from now — if the country develops as it should — this ministry will be among the most important in the government." **Nechemia Meyers**

US telescopes

Caltech takes Texan wind

Washington

PLANNERS of new ground-based optical telescopes in the United States have been given pause for thought since the announcement in January from the California Institute of Technology of plans to build a 10-metre segmented-mirror instrument on Mauna Kea in Hawaii. The Caltech telescope, for which \$70 million has been pledged by the William M. Keck Foundation, will be the largest optical telescope in the world. Fund-raising for other telescopes is likely to be harder as a result, and some plans may have to be rethought.

The Association of Universities for Research in Astronomy Inc. is doing preliminary design work for the National New Technology Telescope (NNTT), an instrument with four 7.5-metre mirrors that would be built by the National Science Foundation (NSF). But it will be difficult to persuade NSF and the Congress to vote \$150-\$200 million of federal funds for NNTT unless it offers US astronomers facilities that are clearly superior to those available elsewhere.

As things stand, NNTT would have twice the light-collecting power and 1.5-2 times the angular resolution of the Keck telescope. The question of whether that factor of two is sufficient to justify spending money on NNTT has yet to be decided, according to Laura Bautz, director of the division of astronomical sciences at NSF, who is forming a committee to examine the questions raised. Caltech is preparing a site for a second 10-metre telescope alongside the Keck telescope which, if it were built, might be combined with the Keck telescope

for optical interferometry; such a combined facility would have characteristics roughly similar to those of NNTT.

Other telescopes have also been put into the shade by the Keck bequest. The University of Texas has, since 1979, been planning a 7-metre single-mirror telescope at its McDonald Observatory in west Texas, but has so far failed to raise the \$50 million needed. The Texas state legislature, which is in the throes of a spending crisis, recently turned down a second request for \$5 million of state funds to purchase the primary mirror for the telescope.

The publicity surrounding the Keck bequest will "undoubtedly make it harder" to find a private foundation willing to support construction of the Texas telescope, according to Harlan Smith, director of the McDonald Observatory. The telescope can no longer tempt potential sponsors with the glory of supporting the biggest telescope in the world (although Dr Smith points out it would still be the largest monolithic mirror telescope). Dr Smith argues that the supply of optical telescopes is not comparable with the supply of accelerators for high-energy physics, where there is continual pressure for ever-higher energies; rather, the pressure on optical telescopes is for observing time.

Even the Texans' claim to be building the world's biggest monolithic mirror telescope may shortly be disproved, however. The University of Arizona is expected shortly to announce a partnership with other institutions to build a 7-8 metre monolithic mirror telescope, and the Mount Wilson Observatory in California hopes for a similar instrument. **Tim Beardsley**

IVF

Australian export service

Canberra

THE *in vitro* fertilization (IVF) technology that produced that first freeze-thaw embryo and donor-embryo babies will soon become commercially available to infertile couples in the United States. The vice-chancellor of Monash University in the state of Victoria announced in Melbourne last week that an agreement had been reached, after 18 months of discussion, to form a private company to establish clinics, train US doctors and make available the techniques developed at the Queen Victoria Medical Centre by Professor Carl Wood, Dr Alan Trounson and their colleagues.

The company will be called IVF Australia (IVFA) and its first clinic is expected to be set up in New York. In the absence of more stringent local laws controlling IVF, the services on offer will conform with the guidelines set out in the Infertility (Medical Procedures) Act (1984), which will be proclaimed by the Victorian State Parliament in the current session, having been deferred early last year when state opposition parties successfully blocked its passage.

The bill specifically forbids surrogate

embryo transfer and the embryo flushing procedure known as lavage (or *in vivo* fertilization), where fertilization is carried out in the womb of one woman and the resulting embryo flushed out and transferred to the womb of another woman. To achieve bipartisan support, the Victorian Labor government was also obliged to deny IVF treatment to couples in a stable *de facto* relationship, except those already embarked on the Monash programme before the act comes into force. This appears to rule out all US *de facto* couples as well as single women.

With more than 200 babies born at a time when most US clinics are closing down, the Melbourne researchers have brought several procedures to a high rate of success. The services offered will initially involve husband-and-wife gametes only, but will probably be extended to donor procedures as well, and will include embryo freezing and storage.

The Victorian legislation is based principally on the recommendations of the Waller committee, set up to consider the ethical and legal issues raised by *in vitro* fertilization (see *Nature* 311, 288; 1984). The

act bans any form of cloning and any procedures where human gametes are mixed with those of an animal. A couple may nominate a (willing) donor, the woman's sister for example, but no donor may receive payment beyond normal expenses.

The Waller committee recommended that research on embryos should extend only over the first 14 days. The Victorian government has not yet formulated a full reply to the committee's recommendations but the draft legislation provides for a Standing Review and Advisory Committee to be created to consider requests for approval of research with embryos.

Where does the act leave the frozen Rios embryos (see *Nature* 309, 738; 1984), whose biological parents perished in an air crash, leaving no instructions for their ultimate disposition? According to the draft Victorian legislation as it now stands, the embryos would be made available to other couples on the IVF programme who would be told that the embryos are unlikely to grow to maturity because the methods used to freeze them were not as well developed as those now available. The prospective social parents would be supplied with non-identifying information about the biological parents (the Rios) and if a person grown from one of the Rios embryos were to seek this information from the Victorian State Health Commission, it would also be non-identifying, subject to the discretion of the minister.

No development or discovery will be incorporated as part of IVFA's programme until approved by a Bioethics Committee which, in the manner of the Waller committee and the Standing Review and Advisory Committee, is likely to consist of lawyers, philosophers, social workers and medical researchers. The company will not for the time being have its own research and development function; the Monash University team, however, will continue to publish its research results. Where patentable discoveries emerge, care will be taken to enable proprietary rights to be secured but, under the agreement, patenting of medical procedures will not be permitted. The university has no equity in the company but will receive royalties. Members of the IVF team in Melbourne will remain employees of the university and may receive payment on a consulting basis.

Professor Wood says that the money for research generated by the royalty payments has become necessary to keep the team together, given the low support accorded to the IVF programme in the past; ethical objections to profit derived from the advanced techniques would, if taken up, appear to apply to all private medicine. There could be no absolute guarantee, he says, that a US doctor trained in the new techniques would not start his own clinic offering procedures illegal in Victoria, but such a clinic would then not continue to enjoy support from the Melbourne IVF team, and a personal vetting scheme would also help to minimize this risk. **Jeffrey Sellar**

Hetero-solid growth

UK seeks to catch up

A MULTI-MILLION pound stimulus to British research in "low dimensional structures" over the next five years has been announced by the Science and Engineering Research Council (SERC). The programme is intended to enhance the United Kingdom's ability to compete scientifically and, in the longer term, industrially in the field of heterojunction semiconductors, and the new physics associated with such devices. SERC has already allocated £3.5 million to the scheme, which forms a part of the strategy for "core science" heralded by the council's Science Board last year (see *Nature* 309, 388; 1984).

Interest in low-dimensional structures has burgeoned over the past few years with the realization that they offer unprecedented opportunities for the study of semiconductor physics. In particular, the techniques of molecular beam epitaxy (MBE) and metal organic chemical vapour deposition (MOCVD) permit the construction of interleaved layers of semiconductor materials of precisely determined thickness — in many cases, only a few angstroms. In a superlattice, for example, layers of GaAs may alternate with GaAlAs. The difference between the two materials in their respective energy gaps separating the valence and conduction bands can result in a two-dimensional layer of highly mobile electrons. The physics of such layers, in which unique phenomena such as fractional quantum effects and phase transitions have

been observed, is one of the most active research areas, while the potential industrial spin-off with, for example, high-speed devices and optical communications and logic is vast.

To a considerable extent, British researchers have, until recently, had to confine their studies to "off the shelf" materials supplied by companies such as GEC, Philips and Plessey. As a result of the new initiative, however, university centres for material growth are being established, allowing researchers not only to design and grow materials as dictated by their research but also to study and improve the growth techniques themselves. For instance, a group at the University of Cambridge, with the collaboration of GEC and British Telecom, is developing *in situ* electron lithography during growth by MBE.

MBE work will also be carried out at the University of Nottingham and Imperial College, London, while an MOCVD facility is being developed at Oxford. A typical cost for the establishment of an MBE or MOCVD facility is anything up to £500,000. Industrial collaboration will involve part-sponsored studentships and funding of cooperative research grants. In such schemes, the company involved generally has right of first refusal of patentable discoveries, proceeds being shared with the public purse through the British Technology Group.

Philip Campbell

NIH research grants

Multi-year grants on ice

Washington

THE Reagan administration's Office of Management and Budget (OMB) has struck a compromise with leaders of the Republican-controlled Senate over the number of new research grants to be awarded by the National Institutes of Health (NIH). According to the agreement, the number of such grants awarded each year would settle at around 5,500, 10 per cent above the current level but considerably below the 6,500 foreseen last year by Congress.

The agreement, if accepted on the floor of the House of Representatives and of the Senate, might defuse the confrontation with the White House over OMB's scheme to circumvent the wishes of Congress by instructing NIH to hold back \$200 million of the \$938 million that had been intended for this year's grants (see *Nature* 313, 377; 1985). The issue of NIH grants remains controversial in Congress, however, and the agreement of both houses is by no means assured.

Congress, ever-generous to NIH, last year voted it a large budget increase and urged increasing the number of new and competing research grants awarded each year from 5,000 to 6,500. Because grants last typically for three years, however, Congress's action would have meant further large increases for the NIH budget in 1986 and 1987. OMB, seeking to reduce the federal budget deficit, told NIH to disregard the new grant figures suggested by Congress and to hold the number steady at 5,000; the \$200 million left over as a result should be used to support second- and third-year grant continuations in 1986 and 1987.

The legal basis for OMB's action was

questioned last month by the US Comptroller General, who was responding to a query by Senator Lowell Weicker. The Comptroller General's opinion was that the executive branch was not legally obliged to follow the wishes expressed in congressional appropriation committee reports about the number of grants NIH should support; on the other hand, OMB's specific plan, to fund 646 multi-year grants with the 1985 appropriation, was found to be illegal.

The administration has challenged the Comptroller General's opinion, and in the meantime the \$200 million in question is being held in reserve: the matter has been referred to the Justice Department for a second opinion. As a compromise, the administration has undertaken that NIH should not commit funds to any multi-year grants before 1 July, by which time the question of legality will presumably have been sorted out.

Last week's agreement with Republican leaders refers formally only to fiscal year 1986 and beyond; it does not address the problem of what to do about this year's appropriation. It is thought likely, however, that the same figure of 5,500 new grants might be an acceptable compromise for this year as well. The administration would nevertheless still like to establish the legality of multi-year funding, despite certain opposition from Congress. Should legal obstacles prevent this, all the \$200 million now being held back would have to be spent this year. One possibility being explored is that it might be used for a one-time-only extension of grants that narrowly failed to be extended this year: this would placate many disappointed researchers without incurring the commitment of a new three-year grant.

Tim Beardsley

UK embryo research

Government stops Powell bill

The British government has now decided effectively to block the Unborn Children (Protection) Bill in its passage through the final stages of procedure in the House of Commons. The bill, which seeks to prevent the use of *in vitro* fertilized embryos for purposes other than to bring to term a particular fetus, was introduced by Mr Enoch Powell, MP, and received a large majority (127) at its second reading (see *Nature* 21 February, p.618). On 3 May, when the bill is due to be referred back to the House of Commons from committee, the government has decided not to provide enough time for debate, so that the bill's opponents will be able to talk it out. This decision has infuriated supporters of Mr Powell's bill, who argue that it should receive government backing because of the large percentage of MPs in favour of it.

A Marplan opinion poll published in

Britain this week, however, reveals that 32 per cent of adults are in favour of embryo research, 41 per cent are against and 27 per cent "don't know". Nearly half of the opponents changed their minds when asked if they would favour research if it would help to eliminate genetic diseases, resulting in a total of 51 per cent in favour of research.

The government plans to introduce its own legislation, possibly in the parliamentary session beginning next October, based on the findings of the Warnock committee (see *Nature* 312, 389; 1984). This legislation will be more comprehensive than Mr Powell's bill; Mr Norman Fowler, Secretary of State for Social Services, is pledged to prevent commercial surrogacy agencies of any kind from operating in Britain, and this will be included in the government's plans.

Maxine Clarke

Algal structures



FIXED algae have been used for many purposes, but rarely for making what the French sculptor Ernest Pignon-Ernest calls "arbrorigènes". This pun on the French words for "tree" (*arbre*) and "aborigine" describes a collection of life-sized models carved out of polystyrene and containing fixed *Porphyridium cruentum*, a reddish single-celled alga that gives the polystyrene flesh tones. Pignon-Ernest's models now adorn the trees of the Paris Jardin des Plantes. The sculptor is fascinated by the idea that these sculptures need water and light.

Pignon-Ernest is not a scientist. He conceived the idea of using fixed algae, however, and approached biotechnologist Daniel Thomas of the University of Compiègne and his colleagues Claude Gudin of the solar biotechnology laboratory at Cadarache for ideas. Thomas, who specializes in cell and enzyme-fixing, had the technology of fixing *P. cruentum* (a common garden species) in polystyrene, and Gudin has been using it to produce polysaccharides using solar energy. (When its environment dries, the organism protects itself by secreting a sugary shell.) Pignon-Ernest found the material ideal, and the "breathing" arbrorigènes were the result.

Thomas, meanwhile, was also at work with another artist, Monet — or at least with his enormous "water-lilies", a 186 sq. m. canvas hanging in the Louvre gallery in Paris. This painting was damaged when it was first transported. Paper was glued to the surface of the paint to protect it, but the wrong glue was used and the picture remained misted and in danger of denaturing by interaction of glue and paint. Thomas came to the rescue, discovered the glue was starch-based and dissolved it off with enzymes. Which proves that biotechnology has its uses, even if those uses will have little effect on share prices...

Robert Walgate

Name for AIDS virus

SIR — Although I personally believe that the virus associated with acquired immune deficiency syndrome (AIDS), HTLV/LAV, has enough structural and functional similarities with the other human T-lymphotropic viruses (HTLV-I and HTLV-II) to justify its classification within the same family¹, I also recognize that more than just science and logic are at stake over the recent nomenclature debate². If a more non-partisan name is ultimately called for, my personal suggestion is that the virus should be called human AIDS/lymphotropic virus (HALV). Human, because in the tradition of naming retroviruses, the species name is given; AIDS, because although it is not the only disease this virus causes, it is the one that gives it notoriety; lymphotropic, because this is the most basic and neutral characteristic of the virus. Since it looks enough like HTLV, and contains all three letters of LAV, it may well be the perfect solution for the two groups to meet *halv* way.

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1. Wong-Staal, F. & Gallo, R.C. *Blood* 65, 253-263, (1985).
2. Marx, J.L. *Science* 227: 1449-1451, (1985).

Aid for Chile

SIR — As you probably know, we in Chile are at present in great distress. On the evening of 3 March, Santiago suffered a catastrophic earthquake. An international campaign has already started, mainly to solve the immediate needs of the several hundred thousands of homeless people. The building of our physics department, although still standing, was severely damaged, most probably beyond repair.

Science in general and physics in particular are vulnerable in developing countries and clearly more so in situations like the present one. We scientists are often looked upon with suspicion and we cannot at present expect any significant help from the authorities.

Nevertheless, we have not lost our optimism and desire to carry on with our work. After assessing the real effects of the earthquake on our building we have decided to start a campaign to raise funds in order to build up a new one. We estimate that the basic need of the thirty physicists in our department could be met by a twenty thousand square foot construction.

We would welcome any material help or advice that our colleagues in the scientific community can offer.

LINCOYÁN GONZÁLEZ

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Embryo research

SIR — I am disgusted with your call for research ideas on human embryos (*Nature* 7 March, p.11). Any researcher participating in it (and those experimenting on human tissue obtained at abortions) should be forced to read Trevor-Roper's book review "Seas of unreason" (*Nature* 313, 407; 1985).

The primary objective for any physician has to be care for the individual patient. We must not sacrifice human individuals for a potential benefit to others. Just as we do not kill AIDS patients to prevent the infection of others, experimentation with and killing of embryos and fetuses cannot be justified by potentially interesting research results. A statement of what those benefits might be is therefore irrelevant.

If human societies ever become aware again of those hippocratic truths, many of today's researchers will be judged then as are Third Reich doctors today. And we will ask disgustedly again, in the words of Trevor-Roper: "How could a civilized nation, in the twentieth century, not only permit it but actually cooperate in it?"

CHRISTOPH BODE

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SIR — I read the News and Views article on human embryo experimentation (*Nature* 7 March, p.11) with incredulity.

Those of us who are not wholly in agreement with Enoch Powell's bill may believe that there are circumstances in which extreme necessity may make experiments of this type allowable. But your anonymous columnist is inviting scientists to *invent* research to do upon embryos: to dream up ideas, which might not otherwise have occurred to them, for research projects which would not otherwise have taken place.

Surely the ethical dubiousness of this incitement to experiment can only strengthen public opposition to all such experiments?

R.J. BIRD

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Suitable treatment

SIR — In a recent leading article about the unauthorized implant of an artificial heart, *Nature* describes the stereotypes being impressed upon the surgeon and the Food and Drug Administration (FDA) as the rugged individualist and the pin-headed bureaucrat. In the same vein *Nature's* role could be described as the aloof and bloodless intellectual. Stripped of its urbanity, what your editorial really said was: let the patient die. *Nature* admits the implant functioned, and that the proper role for future implants may indeed be to stabilize patients awaiting appropriate transplants. Your objections to its use are very thin.

The issue of informed consent seems to me to be very artificial. Informed consent had been obtained in advance for a very ag-

gressive procedure, a transplant. Do you really feel that even in the calm period before the surgery the patient and family would have objected to the possible use of an artificial heart on a temporary basis? The issue of FDA approval would seem very abstract and irrelevant to a family who felt that the implant had a good chance of working for the short time required.

The issue of use of a non-approved device should be put into perspective. Use of drugs for purposes not approved by the FDA is common practice in the United States. A good example is xylocaine, which is approved only as an anaesthetic, but has proved very valuable in controlling certain cardiac arrhythmias. It has been used effectively and apparently safely in that mode all over the country, and no one has said a word. Physicians use it, in some cases, because it saves patients who might otherwise die. Artificial hearts have captured the public imagination and so have engendered intense scrutiny of their use. The issues are no different from those dealt with in a more flexible way every day.

The idea that the surgical team could have been prepared with an authorized back-up is ludicrous. The cost would be astronomical, and the personnel and time are not there. The fact is that FDA has authorized a procedure, the transplant, without the possibility of the implant back-up that may be needed. Placed in this impossible situation different surgeons will react in different ways. I would prefer my surgeon to be the rugged individualist not dominated by the pin-headed bureaucrat. *Nature's* aloofness, either at the bedside or funeral, would not be welcome.

EATON E. LATTMAN

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Dinosaurs did well

SIR — I am a layman, but I am tired of hearing about "theories" (really mere conjectures, as they cannot be tested) of the extinction of the dinosaurs.

They ruled the roost for a hundred million years, ample time for the opposition to get its act together, for the evolution of small, elusive animals that destroyed vegetation and ate the dinosaurs' eggs so that they died of hunger faster than they could reproduce.

They died out "suddenly" did they? How can we know? For any traces to survive, the animal had to die in very special circumstances, so the absence of evidence proves nothing. And how many hectares of the Earth have been excavated?

This kind of nonsense may be good clean fun for the palaeontologists; but people in the media seem to think that I wish to hear of it. I do not.

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Origins of five-fold symmetry

That solids with five-fold symmetry may exist seems plausible enough, but a brief bout of calculation serves chiefly as a proof of how much more remains to be done.

LAST year's gust of excitement at the recognition of five-fold symmetry in a binary alloy (of Al and Mn) by Shechtman, Blech, Gratiyas and Cahn (*Phys. Rev. Lett.* **53**, 1951; 1984) has already spawned its own mini-industry. The surprise, then, was that there should be evidence of any kind for five-fold symmetry when this is strictly forbidden, on geometrical grounds, in a crystal with regular translational periodicity (see *Nature* **313**, 263; 1985).

By the end of the year, D. Levine and P.J. Steinhardt had produced an outline explanation of how such a structure could exist (*Phys. Rev. Lett.* **53**, 2477; 1984). They showed how to construct "quasi-crystalline" structures in two or three dimensions which, although devoid of translational symmetry, could have local five-fold symmetry. Now attention has turned to the question whether such structures will be energetically preferred to regular crystals. But the clutch of three papers published (in *Phys. Rev. Lett.* **8** April 1985), all of which follow the same track, show such discordancies as to suggest that the issue is far from settled.

The starting point is the Landau theory of the evolution of symmetry by means of phase transitions from, say, a liquid in the process of solidification, or one solid phase in the process of transition to another. The trick, following Landau, is to suppose that the free energy is a function of parameters which describe the degree of geometrical order in the system. The first task is to determine what order parameters are allowed by geometry.

Everybody agrees that the order parameters to use are quantities D_k which are the coefficients of mass-density waves within the aggregated system, and which turn up in mathematical expressions of the form $D_k \exp(i\mathbf{Q}_k \cdot \mathbf{R})$, where $\mathbf{Q}_k$ is some fixed vector and $\mathbf{R}$ is the vector representing position. (The symbol i is the square root of -1 .) This, of course, is a periodic function of a kind that might describe plane waves travelling in direction $\mathbf{Q}_k$.

A single order parameter, associated with a single vector $\mathbf{Q}$, suffices to describe the smectic phase of a liquid crystal (in which molecules assume a periodic arrangement in only one dimension within the medium in which they are dissolved). The ordering of regular crystals, on the other hand, requires at least three order parameters, each of them associated with a different vector $\mathbf{Q}_k$. These must jointly describe how it is possible to choose an infinite number of

directions within the crystal in which identically constituted layers of atoms are periodically repeated (and which are defined, in crystallography, by the Miller indices hkl). The basic vectors describing these recurring planes of atoms are not those defining the translational symmetry of the crystal, represented by the repeated translation of the unit cell, but rather the vectors of what is called the reciprocal lattice. All this is standard textbook stuff.

The neatness of the Landau treatment stems from its representation of the free energy as a power series expansion in terms of the mass-density distribution, itself the sum of terms such as $D_k \exp(i\mathbf{Q}_k \cdot \mathbf{R})$ taken over all possible vectors $\mathbf{Q}_k$ in the reciprocal lattice. The separate terms are themselves harmonics of similar expressions involving only the basis vectors of the reciprocal lattice.

Structures with the symmetry of a body-centred cubic lattice are thus signalled by terms which are products of six D -coefficients, each corresponding to one of the six independent vectors of the tetrahedron which is the unit structure of the reciprocal lattice. Face-centred cubic symmetry is similarly signalled by terms of the eighth power, where the corresponding vectors are those of the unit octahedra of the reciprocal lattice. Telling whether a melt will crystallize in one form rather than the other may then simply be a matter of guessing whether one form of symmetry will give a smaller free energy than the other, which in practice entails much arm-waving.

The novelty now reported is that the same argument can be used to judge the likelihood that symmetries not allowed in ordinary crystallography will occur. Per Bak from the Brookhaven National Laboratory puts the case most simply by enumerating the terms in a Landau expansion corresponding to five-fold symmetry (*Phys. Rev. Lett.* **54**, 1517; 1985). The simplest case is that of fifth-power terms (products of five D -coefficients) corresponding to the five vectors represented by the sides of an equilateral planar pentagon, but this corresponds in three dimensions merely to a structure built of parallel rods whose cross-sections are the elements of the Penrose tiling of the plane.

Bak gets true five-fold symmetry by picking out the Landau terms corresponding to the null combinations of the fifteen pairs of vectors along the edges of a regular icosahedron. These show up as ten third-degree terms and six fifth-degree terms,

corresponding to the ten triangles and five pentagons that can be drawn on the surface of an icosahedron. Bak readily concludes that a structure with five-fold symmetry can be stable relative to body-centred cubic structures if only the parameters are suitably adjusted.

Levine and Steinhardt (see above), together with a number of colleagues at the University of Pennsylvania and John Toner from the IBM research centre, follow a similar tack but calculate the elastic constants of a solid with five-fold symmetry to demonstrate its stability (*Phys. Rev. Lett.* **54**, 1520; 1985). As in the simple Landau treatment, symmetry restricts the range of terms occurring in the equations, but this line of argument has the virtues of showing how dislocations may be introduced into structures with five-fold symmetry (by means of pretty pictures made by driving a dot-matrix printer by the expressions calculated for the mass-density distribution) and of making explicit the hidden symmetry of these perplexing five-fold structures, which emerge as the representation in three dimensions of much simpler structures in six dimensions.

So far, there is very little physics in these discussions, but M.D. Mermin and Sandra M. Troian from Cornell University have a novel twist (*Phys. Rev. Lett.* **54**, 1524; 1985). They say that they find only metastable states for a solid with one component and with five-fold symmetry (but they appear not to have included fifth-degree terms, corresponding to the six pentagons on the surface of an icosahedron, in their Landau calculation). But how relevant is it that the only real solid in which five-fold symmetry has been found so far is an alloy, with two components?

In an alloy, there is scope for a dimension of disorder beyond those specified by the wave vectors of the simple Landau calculation. On this view, the argument goes, there is scope for generating five-fold symmetry from a blend of geometrical shape and occupancy by one or other of two components. So far as it goes, the argument is suggestive only, although the most telling evidence is a pair of dot-matrix diagrams, said to represent a cross-section from a structure with five-fold symmetry, in which one component almost exactly fills the gaps in the distribution of the other. Time, and some tangible experimental investigation, will no doubt tell whether this is what five-fold symmetry is like.

John Maddox

Molecular Egyptology

Mummified human DNA cloned

from J.S. Jones

ON page 644 of this issue, Svante Pääbo reports the remarkable extraction and cloning of DNA from an Egyptian mummy¹. One clone contains a 3.4 kilobase segment of DNA that has been preserved for more than two millennia. Although only one out of 23 mummies has yet produced clonable DNA, the yield from this individual approaches five per cent of that obtainable from fresh human material. Preliminary though it is, the report opens up some fascinating possibilities for future work on human evolution and archaeology.

'Fossil genes' have been identified before now: ancient snails buried in prehistoric English earthworks retain their shell colour and banding patterns², and comparisons of skeletal variants in living and extinct mice have been used to establish the origins of some Scottish mouse populations³. Even mummies retain enough of their blood group substances to allow the kinship of family groups to be confirmed serologically⁴ and, on the basis of hair characters, an unidentified royal mummy from the New Kingdom has been shown to be the mother of the heretic pharaoh Akhenaton⁵.

Valuable as such specific data may be, there is much more potential information in DNA sequences. The human genome possesses hundreds of thousands of genetic polymorphisms at the DNA level. Some regions (which involve variations in the number of copies of repeated sequences not dissimilar to the *Alu* sequence, to which the mummy 3.4 kilobase clone is closely related) are hypervariable⁶ and can provide particularly precise estimates of human kinship and population relatedness. If these polymorphisms can be detected in mummies, we may answer some important questions about the world of our ancestors.

Information on DNA sequences is uniquely suited for tracing population movements: knowledge of the geographical distribution of point mutations in some senses provides no more than single letters in a population's genetic identity, while the ordered sequence of bases provided by its DNA haplotype gives the genetic surname itself. Thus, information on DNA sequences closely linked to the sickle-cell haemoglobin polymorphism has recently been used to establish the relationships between living populations in different parts of Africa⁷. Sickle-cell populations from Senegal and Benin differ considerably in their sickle-cell haplotype; but populations from North Africa have the same haplotype as the Nigerians. This is strong evidence that the sickle-cell mutation has arisen several times, but that the northern sickling populations originated by migration from West Africa.

Information of this kind from mummies

might tell us something about population movements in ancient Africa. Ancient Egypt is thought to have been occupied by a number of distinct populations, and it is certainly the case that some dynasties of Pharaohs were drawn from foreign populations that invaded the Nile Valley. Perhaps it would be possible to test the claim of the Coptic Christians of modern Egypt to be the lineal descendants of the ancients (who disappeared from the Nile Valley under successive waves of invasion by Syrians, Greeks, Romans, Byzantines and Arabs).

DNA sequences are also useful in studying patterns of kinship and paternity within local populations. There is considerable controversy among Egyptologists about the relatedness of groups of mummies from particular sites, and there is clear potential for DNA work here. Ancient records suggest that in the 18th Dynasty, the Egyptian royal family practised incest between brother and sister or father and daughter for at least eight generations: information on their mummified DNA sequences might tell us to what extent this royal exclusivity was successful. Incest in Egypt was designed to preserve the blood line of a deity. There is something intriguing about the possibility of learning the genotype of a god.

Mummies are not unique to Egypt: preserved humans have been found in Peru, Japan, Australasia and Europe. There is the potential here to answer some larger questions about human evolution. What is the relationship between the native Australians and the rest of the world; were

there indeed two separate invasions of Australia, as often claimed? Do the genotypes of mummified Europeans accord with the suggestion that geographical patterns of gene-frequency change in modern Europeans reflect an invasion from the East that occurred as a result of the development of agriculture? Mr Average, as defined by the population nearest the global mean for a large number of enzyme and blood-group loci, lives in the Middle East⁸: would the patterns of change in mummified humans support the idea of worldwide spread from the Middle East? An answer to any of these questions would be of extraordinary interest.

Finally, it is important to understand that Pääbo has *not* done: we cannot of course reconstitute a functional gene (let alone a living individual) from this short repeated sequence. One American newspaper reported the recent cloning of mitochondrial DNA from a specimen of the extinct quagga⁹ as "US Scientists Clone Dinosaurs to Fight on after Nuclear War", and probably there will be similar sensational accounts of mummy DNA. Molecular biology rarely lives up to its headlines, but the interaction of DNA technology and archaeology may open a new phase in our understanding of human history. □

1. Pääbo, S. *Nature* 314, (1985).
2. Cain, A.J. in *Ecological Genetics and Evolution* (ed. Creed, R.) 65 (Blackwell, Oxford, 1971).
3. Berry, R.J. *Symp. zool. Soc. Lond.* 26, 3 (1970).
4. Harrison, R.G. *et al. Nature* 224, 325 (1969).
5. Harris, J.E. *et al. Science* 200, 1149 (1978).
6. Jeffreys, A.J., Wilson, V. & Thein, S.L. *Nature* 314, 67 (1985).
7. Pagnier, A.J. *et al. Proc. natn. Acad. Sci. U.S.A.* 81, 1771 (1984).
8. Piazza, A.A. *et al. Proc. natn. Acad. Sci. U.S.A.* 78, 2638 (1981).
9. Higuchi, R. *et al. Nature* 312, 282 (1984).

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Archaeoastronomy

Halley's comet in Babylonia

from C.B.F. Walker

IN August 1984, Halley's comet was unexpectedly discovered in the British Museum or, to be more precise, on some Babylonian tablets in the museum's archives. That discovery, reported by F.R. Stephenson, K.K.C. Yau and H. Hunger on page 587 of this issue, will be exciting both for eager Halley watchers and for historians of science. It represents the first significant addition to our knowledge of the past history of the comet since the French publication of Chinese observations in 1846. It fills an important gap in the historical record, since no other account of the return of the comet in 164 BC survives. Remarkably, it also proves that the Babylonian astronomers kept records of such accuracy that we can now make a clear choice between current theories of the

comet's path in antiquity.

What were the Babylonians doing recording comets? The beginnings of astronomy in Mesopotamia probably go back to the third millennium BC, when the Sumerians gave the planets and principal constellations their names but, apart from simple tables of the length of daylight and the rising and setting of constellations, almost all the early texts are concerned with astrology rather than astronomy. Writing in about AD 150, the Greek astronomer Ptolemy notes that the earliest observations available to him date from the time of the Babylonian king Nabonassar (Nabu-nasir, 747-734 BC). Although contemporary texts do not survive, later Babylonian texts also suggest that the practice of keeping a regular official record on the movements

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Babylonian cuneiform tablet (BMA 41462) containing a record of Halley's comet in 164 BC. (Photograph courtesy of The British Museum).

of the Moon and planets began in Nabonassar's time. The surviving texts from Babylon number rather more than 2,000, most of which are now in the British Museum. Among these, some 1,200 are fragments of observation records, a type known as 'astronomical diaries', mostly datable to between about 380 and 40 BC. Others are of a strictly mathematical character, empherides for the moon and planets. The latest text that can be dated is an almanac for AD 75.

The astronomical diaries give a day-by-day account of lunar and planetary observations and weather conditions, with monthly summaries of the height of the Euphrates, prices of various staple commodities, political events, and occasional references to comets and earthquakes, the basis for detailed mathematical systems for predicting recurrent phenomena.

The fundamental concern of the Babylonian astronomer was with the movements of the Moon and planets about the ecliptic. The fact that they moved in a precisely defined belt of stars was recognized early on, and the constellations through which they moved (at first eighteen, later reduced to twelve) were already listed in about 1,000 BC. There is a curious dichotomy between observation and theory in Babylonian astronomy. Texts of observations record the movements of the Moon and planets with reference to a list of 31 stars of varying brightness lying at unequal intervals along or near the ecliptic; distances from the 31 stars are measured in cubits (kuš), which appear to have a variable length of either 2 or $2\frac{1}{2}$ degrees. The theoretical texts, ephemerides and almanacs, which first appear late in the fourth century BC, use the familiar zodiacal system of twelve equal constellations, each divided into 30 degrees (uš).

This dichotomy is also reflected in the

astronomical diaries. Although these ostensibly record observations, analysis shows that all phenomena such as the equinoxes and solstices, the appearances of Sirius, and the stationary positions of planets are in almost every case derived from theoretical schemes rather than from observations.

Our primary source for the identification of the constellations is a text known as Mul-Apin, the 'Plough'. This lists the stars in various groups or 'paths' with details of their heliacal risings and settings. Other texts give descriptions of the appearance of the constellations, and there is also a very small group of drawings. At least in the zodiac belt, the constellations correspond

largely to the picture familiar from the detailed descriptions of Ptolemy, but there are some interesting differences. For instance, Aries is not a ram but a 'hired worker'. More significantly, and still not fully explained, the zodiacal system does not start at the First Point of Aries, 0° ecliptic longitude, but at about 355° , a difference that extends through the whole zodiac.

In texts of observations, measurements of latitude are expressed as so many cubits 'above' (to the north) or 'below' (to the south), relative to a star or planet. Only in the lunar ephemerides is there a concept of absolute ecliptic latitude.

For chronology, the astronomical texts use the king's regnal year (or, from 311 BC onwards, the Seleucid era), the lunar month — beginning with the first sighting of the new moon — and the day. The Babylonian day began at sunset and, for astronomical purposes, day and night were divided into three watches each. Water clocks were used to time observations, the units of time being the bēru (2 hours) and the ūš (four minutes). A group of 26 stars culminating at the zenith provides an additional reference point.

Edmund Halley, in the first publication of his discovery of the orbits of comets, begins by referring to a story of the classical historian Diodorus Siculus that the Chaldeans (Babylonians) kept records of storms, earthquakes and comets, and tried to predict their occurrence. He dismisses the story as being of interest only to astrologers. Even thirty years ago we had little idea of how well informed Diodorus really was. Halley would be astonished to know how valuable the Babylonian records of comets have come to be for the study of his own comet. □

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Biotechnology

New route to antibiotic creation

from Arnold L. Demain

ALTHOUGH developments in recombinant DNA (rDNA) technology have not escaped the attention of the antibiotic industry, the report on page 642 of this issue from a collaboration of British, Japanese and American laboratories that novel antibiotics can be obtained using the technique is a major step forward in biotechnology¹.

The rapid movement of rDNA technology into secondary metabolism can be mainly attributed to the long-term dedication of David Hopwood and his colleagues at John Innes Institute in Norwich to the genetics of *Streptomyces* species. Having developed plasmid and phage vectors for applying rDNA technology to these filamentous bacteria, in 1983 the Hopwood group cloned the gene of the methyltransferase of undercylprodigiosin biosynthesis in *Streptomyces coelicolor*. (Prodigiosins are pigmented antibiotics produced by unicellular and filamentous bacteria). Independently, Williams and colleagues in Houston cloned and expressed prodigiosin biosynthetic genes of *Serratia marcescens* in *Escherichia coli*³. The next developments were the cloning of the *Streptomyces*

griseus *p*-aminobenzoic acid synthase gene of candicidin biosynthesis in *Streptomyces lividans*⁴ at Norwich and that of a gene of clavulanic acid biosynthesis from *Streptomyces clavuligerus* at the Beecham Laboratories⁵. Finally, the Hopwood group accomplished the cloning of a segment of chromosomal DNA from *S. coelicolor* that carries the complete information necessary for the biosynthesis of the antibiotic actinorhodin⁶. Also of interest is the recent cloning of a *S. coelicolor* regulatory gene controlling the synthesis of actinorhodin, undecylprodigiosin and A-factor (a differentiation effector in streptomycetes)⁷.

In order to enhance the probability of success in their attempts to produce hybrid antibiotics, Hopwood *et al.* employed microbial cultures producing three pigmented antibiotics of the isochromanquinone class. The cultured organisms were *Streptomyces coelicolor* A3 (2) (which produces the antibiotic actinorhodin), *Streptomyces* sp. AM-716 (medermycin) and *Streptomyces violaceoruber* Tü22 (granaticin). Two hybrid antibiotics — mederrhodins A

and B — were produced following the introduction of *S. coelicolor* actinorhodin biosynthetic genes (originally located on a chromosome, but placed on a plasmid vector) into *Streptomyces* sp. AM-7161. Mederrhodin A was identified as hydroxymederrhodin. Cloning of *S. coelicolor* DNA in *S. violaceoruber* yielded a third hybrid antibiotic, dihydrogranatirhodin, which was found to be the C-3 or C-1 epimer of dihydrogranaticin. Evidence is put forth that each of the novel products are produced by genetic contributions from both recipient and donor microorganisms rather than merely by activation of silent genes of the recipient.

It should be noted that this is not the first technique developed for the production of hybrid antibiotics. They have been produced by 'mutational biosynthesis' for about 15 years⁸. In this technique, an antibiotic-producing strain is first mutated so that it is blocked in forming a moiety of the antibiotic and is then fed with moieties of other antibiotics. The technique has yielded hundreds of novel antibiotics but unfortunately none has become commercially important. A second method has involved natural mating between different actinomycete strains or species⁹⁻¹¹, and seems to produce new antibiotics by activation of latent genes in the recipient strain¹². A third method involves protoplast fusion between strains producing different antibiotics¹³.

Since new antibiotics are going to be required for many years to come in order to counteract the development of resistance in infectious microbes and the existence of diseases still unconquered by available compounds, antibiotic creation by rDNA technology is a welcome addition to existing techniques. But in case any group jumps to the erroneous conclusion that conventional efforts are now superceded, it should be understood that the rDNA technique is only a tool to be added to those of soil-screening, mutational biosynthesis and protoplast fusion. In our continuing struggle against infectious disease every new technique is welcome. □

1. Hopwood, D.A. *et al.* *Nature* **314**, 642 (1985).
2. Feitelson, J.S. & Hopwood, D.A. *Molec. gen. Genet.* **190**, 394 (1983).
3. Dauenhauer, S.A., Hull, R.A. & Williams, R.P. *J. Bacteriol.* **158**, 1126 (1984).
4. Gil, J.A. & Hopwood, D.A. *Gene* **25**, 119 (1983).
5. Bailey, C.R., Butler, M.J., Normansell, N.D., Rowlands, R.T. & Winstanley, D.J. *Bio/Technology* **2**, 808 (1984).
6. Malpartida, F. & Hopwood, D.A. *Nature* **309**, 462 (1984).
7. Horinouchi, S., Hara, O. & Beppu, T. *J. Bacteriol.* **155**, 1238 (1983).
8. Daum, S. & Lemke, J. A. *Rev. Microbiol.* **33**, 241 (1979).
9. Ihn, W., Schlegel, B., Fleck, W.F. & Sedmera, P. *J. Antibiot.* **33**, 1457 (1980).
10. Mazieres, N., Peyré, M. & Penasse, L. *J. Antibiot.* **34**, 544 (1981).
11. Traxler, P., Schupp, T., Führer, H. & Richter, W.J. *J. Antibiot.* **34**, 971 (1981).
12. Hopwood, D.A. in *Biochemistry and Genetic Regulation of Commercially Important Antibiotics* (ed. Vining, L.C.) 1 (Addison-Wesley, Reading, Massachusetts, 1983).
13. Ikeda, H., Inoue, M., Tanaka, H. & Omura, S. *J. Antibiot.* **37**, 1224 (1984).

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Oceanography

Residence times of water masses in regions of the ocean

from John Woods

WHEN a water molecule involved in the global hydrological cycle finds itself in the atmosphere, it is likely to reside there for about ten days. The residence times of water in other components of the hydrological cycle are approximately: Southern Sea ice, a few months; Northern Sea ice, a few years; the World ocean, 3,000 yr; lakes, 100 yr; and the polar glaciers, tens of millennia. These estimates are based either on the ratio of volume-to-surface flux for each component, or on the radioactive age of materials mingled in with the water. There are errors in the estimates, but there is no fundamental difficulty in determining residence times for such distinct components of the hydrological system as the Greenland ice sheet, or the Mediterranean Sea. But problems arise when one tries to subdivide the components further, to discover whether water resides much longer in some parts than others. This is a question of fundamental importance for oceanography, but remains beset with confusion. The problem is first how best to delineate the separate parts and then how to determine their residence times. Equivalent questions arise in systems that range from the cardiovascular system to chemical reactors. The letter by Buffham¹ on page 606 of this issue helps to clarify matters.

Buffham's contribution is to introduce a formalism into the analysis of what he calls "region residence times" of complex circulation systems. The idea is deceptively simple and with hindsight one is tempted to dismiss it as obvious. Yet it is far from trivial and will influence, for example, the way oceanographers study their complex circulation system. What Buffham shows is that the region residence time can be deduced from a model of the system if the volumes of all regions of the model other than the one of interest are reduced to zero and by finding the residence time distribution of the resulting model. He shows that this approach is consistent with previous results and illustrates it with some simple configurations.

The method has potential for the diagnosis of region residence times in models of ocean circulation. A good example of the need for such information is provided by the problem of trying to predict climatic responses to atmospheric pollution by CO₂. Pollution decreases the net infrared cooling of the ocean, which therefore accumulates heat until the surface temperature of the ocean rises sufficiently to rebalance the surface heat budget by enhanced conduction and evaporation. The oceanographer's problem is to predict the global pattern of increasing surface tem-

perature. For that, it is necessary to calculate the distribution of the heat anomaly created by the greenhouse effect. Early models assumed that the anomaly stayed at the surface in a mixed layer 70 metres deep. This assumption produces a characteristic (e-folding) response time of a few years. There are, however, theoretical objections to such models because they neglect the ventilation of anomalies from the mixed layer into the permanent thermocline²; models that include this factor give response times of several decades.

Which, if any, of these predictions is correct could be of great importance for the way that society prepares itself for changes in sea surface temperature, and hence global climate, in response to CO₂ pollution. A slow change would give time to switch to nuclear power, adapt agriculture, build up sea defences, even to move vulnerable populations. If the ventilation of the deep ocean is a factor, then the response may be so attenuated that we can enjoy the agriculture benefits of increased CO₂ production without any unpleasant change in climate. To predict the response accurately requires that the models used have the correct residence times in all the regions through which the thermal anomaly passes. Vigorous efforts are being made to test the ocean models by comparing the distributions of transient tracers in the ocean with those simulated by the models³. The problem is to diagnose the sources of differences, in particular to identify regions in the model that have unrealistic residence times.

That is where Buffham's formalism suggests a research strategy. Of course, ocean circulation is extremely complex and far from being fully understood, but we do know what goes into the models, and sensitivity studies related to residence time can help us to focus on the main issues. Various methods have been proposed for dividing the ocean into layers (for example, Wüst's Kernschicht-Method⁴ and Worthington's isothermal surfaces⁵) but it is now generally accepted that the best division is by isopycnic (constant density) surfaces. Some models are constructed with an isopycnic distribution of grid points⁶. The advantage is that particles circulate around the permanent gyres and are mixed by synoptic-scale eddies along isopycnic surfaces; mixing through the surfaces is very much weaker and can sometimes be ignored. Using isopycnic surfaces ensures that the upper and lower boundaries of the (Buffham) regions can be treated as if they are impermeable, or only slightly leaky.

The lateral boundaries present greater

problems, which are the subject of lively debate in the ocean-circulation community. In general, the periphery of the region is defined by mean streamlines of the large-scale circulation, and by turbulent boundary layers where it meets the seabed or sea surface. These boundary layers tend to vary diurnally and seasonally, and with changing weather and climate. Since the flux of water into a particular region may depend critically on such variations, these have to be taken into account very carefully when calculating residence time.

The climatological problem focuses on the exchange between atmosphere and ocean that occurs through the surface boundary layer. Some regions of the ocean have peripheries that include the surface boundary layer only in winter, and some not every winter. It has been suggested that some regions of the North Atlantic have not been so connected to atmospheric influence since the little Ice Age over a century ago⁵, and parts of the deep Pacific may have been sealed off since the last glaciation. The ventilation of such regions depends on isopycnal diffusion — which

can be estimated accurately; intrusion from the seabed boundary layer — about which we know very little; and slow leakage through the isopycnal surfaces defining the top and bottom of the layer — about which we know even less.

Geochemical dating is vital for estimating the residence time of such regions. They do not necessarily lie only in the depths of the ocean. Theoretical studies have recently suggested they will sometimes be found in the upper thermocline, for example, in the ventilation 'shadow zone' off North-West Africa⁷. The regional residence time problem is central to oceanography. It must be solved before we can expect reliable predictions of climatic change. □

1. Buffham, B.A. *Nature* 314, 606 (1985).
2. Bretherton, F.P. *Prog. Oceanogr.* 11, 93 (1982).
3. Sarmiento, J.J. *Phys. Oceanogr.* 13, 1924 (1983).
4. Wüst, G. *Meteor. Expedition Report Pt 1*, Vol. 2 Berlin (1936).
5. Worthington, L.V. *On the North Atlantic Circulation* The John Hopkins Oceanographic Studies 6 (1976).
6. Bleck, R. & Boudra, D.B. *J. phys. Oceanogr.* 11, 755 (1981).
7. Luyten, J., Pedlosky, J. & Stommel, H. *J. phys. Oceanogr.* 13, 292 (1982).

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Immunology

Immunoglobulin-related domains for cell surface recognition

from Alan F. Williams

IS IT acceptable to categorize cell-surface molecules as recognition structures because they show a clear relationship to immunoglobulin (Igs)? Perhaps not, but one of two newly reported members of the Ig superfamily clearly supports the recognition theme since it is a cell-surface molecule that seems to have an important accessory function in the recognition of antigen by T lymphocytes^{1,2}. The other new structure³ is also found in the T-lymphocyte lineage and it is immediately obvious from the figure and the table that much of the surface of thymocytes and unactivated T lymphocytes is covered with molecules that are now known to be Ig-related.

Human CD8 antigen is a newly-sequenced molecule with a role in T-lymphocyte activation^{1,2}. It is also known as T8 or Leu 2, and corresponds to mouse Ly-2,3 antigen, which was one of the first four T-cell alloantigens described by mouse immunogeneticists⁴. CD8 is found on most thymocytes but amongst mature T lymphocytes it is restricted to those that are precursors for cytotoxic cells (T lymphocytes which kill, for example, virus-infected cells) which recognize, or are restricted by, class I MHC antigens and constitute about 25 per cent of recirculating T lymphocytes⁵.

Antibodies to CD8 antigen can block T-cell cytotoxicity in the recognition phase of the response, so what is the role of CD8

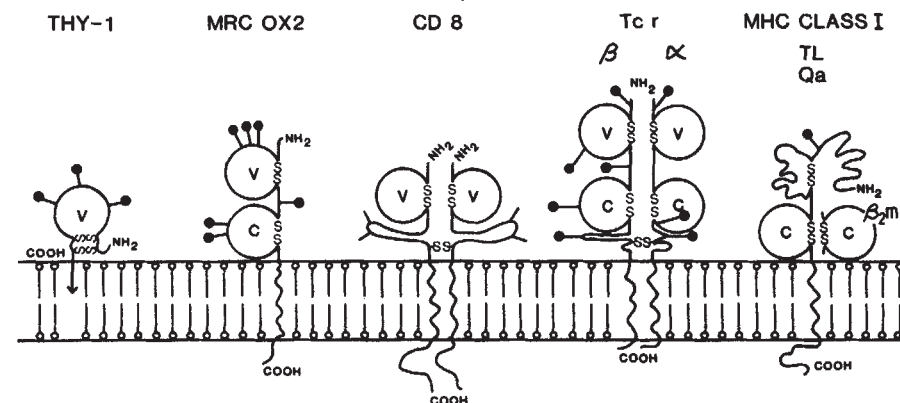
in the cytotoxic reaction? It does not provide determinants that are complementary to foreign antigen but may interact with constant parts of class I MHC antigen to bias cytotoxic cells towards reacting with foreign antigen associated with the class I MHC structure⁶. This could be important if the cells' T-antigen receptors (Tcr), which bind foreign antigen plus MHC antigen, only recognize the polymorphic part of the latter, and so cannot distinguish class I from class II MHC structures. It should, however, be emphasized that an interaction

between CD8 and class I MHC is not established and a clear demonstration of this would be a major advance.

The molecular forms of CD8 antigen are complex and vary between species. Mouse CD8 is a heterodimer consisting of an α - or α' -glycoprotein chain (M_r 38,000 and 35,000), which expresses the Ly-2 allo-antigenic determinant, and a β -chain (M_r 30,000) which expresses the Ly-3 determinant⁷. Rat thymocyte CD8 also has two dissimilar chains, (M_r 38,000 and 34,000)⁸ but human CD8 consists of only one chain (M_r ~ 34,000) which forms a homodimer⁹, although this is further associated with a 45,000 M_r structure on thymocytes. The human antigen was cloned after transfection of mouse L cells with human DNA^{1,2,10}, which led to the cloning of equivalent mouse and rat antigens.

The structural features and relationships of human CD8 are summarized in the figure. Each chain contains one domain that resembles an Ig variable (V) domain, in particular an Ig light-chain V. Following this is a sequence like an Ig hinge, a transmembrane piece and a short cytoplasmic tail. The glycoprotein structure is unusual in having only O-linked carbohydrates⁹ (there is an Asn-Pro-Thr sequence in CD8 but the proline debar N-glycosidic attachment at the asparagine). The larger chain of the mouse heterodimer (Ly-2) is equivalent to human CD8 (L.A. Herzenberg *et al.*, J.R. Parnes *et al.* personal communications) while, surprisingly, the equivalent in the rat is the smaller chain (P. Johnson *et al.* personal communication). The mouse and rat chains have three and one sites for N-glycosylation, respectively, so differences in glycosylation may account for the species variations in size of the chains. The other CD8 glycoprotein chain of rodents remains to be cloned; its sequence will be of considerable interest to the full understanding of a set of molecules that seem to show considerable diversification in evolution.

The second new member of the Ig superfamily is the rat MRC OX-2 antigen,



Models for Ig-related molecules found on thymocytes and/or unactivated T lymphocytes. Circles indicate segments related to Ig domains with similarity to a V-domain (V) or C-domain (C) folding pattern. Double S symbols indicate possible positions of disulphide bonds but these are established only for Thy-1 and MHC class I antigens. N-linked carbohydrate structures (f) are determined by positions of Asn X Thr or Ser in the protein sequence but the number of O-linked structures (l) in CD8 is arbitrary and the positions are guesswork. For Thy-1, see ref. 25; OX-2, ref. 3; CD8, refs 1 and 2; Tcr, ref. 26; MHC class I, TL, Qa, ref. 27.

which is one of the new generation of molecules that has been discovered by means of monoclonal antibodies. This antigen was initially of interest to my colleagues and me because of its expression on thymocytes and neurones, as is the case for Thy-1 antigen¹. OX-2 is also on all endothelial cells, B cells, activated T cells and follicular dendritic cells. There is no evidence concerning its function and no obvious functional correlation between the

cells that express the antigen, except that all may be involved in recognition events. The predicted structure for OX-2, which is included in the figure, shows that it looks remarkably like a single Tcr chain or Ig light-chain with a membrane-spanning segment added. The sequence of OX-2 domain I fits well with the V-domain pattern, perhaps with a closer relationship to Thy-1 than to other sequences.

The Ig-domain hypothesis¹¹ says that

Table 1 Some surface molecules of thymocytes and unactivated T lymphocytes

Molecules	Molecular nature	Commonly-used names			Expression	Comments
		Man	Mouse	Rat		
Ig-related						
T-antigen receptors (Tcr)	gp43; gp40	No antibodies react with all Tcr			Thymocytes, T-helper and T-cytotoxic cells	Tcr seems the same for T-helper and T-cytotoxic cells ²⁶
MHC Class I	Igp43; p12	HLA A,B,C	H-2 K, D	RT1A	Many cells; not brain, not 70% of thymocytes	Recognized along with foreign antigen by Tcr
TL (CD1?)	gp43; p12	NA1/34; T6	TL	NA	Thymocytes and leukaemias	Similar to and genetically linked to MHC I
Qa	gp43; p12	NA	Qa	NA	Lymphoid, others?	Similar to and genetically linked to MHC I
Thy-1	gp18	Thy-1	θ, Thy-1	Thy-1	Neuronal and fibroblasts in all species studied; lymphoid in rodents, not man	Anti-Thy-1 mitogenic for mouse. T cells and affects behaviour of neurons ¹⁸
MRC OX-2	gp47	NA	NA	OX-2	Thymocytes, neuronal, endothelium, follicular dendritic, B cells	Appears before Thy-1 in brain development ¹⁹
CD4	gp55	T4; Leu 3	L3T4	W3/25	Thymocytes, T helpers, macrophages	Antibodies block T-helper response to antigen. Homology to Ig reported in abstract ²⁰
CD8	gp38; gp34 or gp34 × 2	T8; Leu 2	Ly-2; 3	OX-8	Thymocytes, T cytotoxic, NK cells	Antibodies block T-cytotoxic cell reactions
Not Ig-related						
CD3	gp25; gp20; p20	T3, Leu 4	NA	NA	Thymocytes and mature T cells only	Associated with Tcr; antibodies mitogenic if crosslinked at surface; gp20 not Ig-related ¹⁷
L-CA	gp180-240	L-CA	T200, Ly-5	L-CA	All leucocytes but not erythroid cells. Absent from other tissues	Antibodies have functional effects. Sequence shows M_r 80,000 cytoplasmic domain ²¹
LSGP	gp95	L10	NA	W3/13	Thymocytes, T cells, neutrophils, plasma cells, brain	Mucin-like, peanut lectin receptor; deficiency may cause Wiskott-Aldrich syndrome ²²
LFA-1	gp180; gp90	LFA-1	LFA-1, Ly-15		Most leucocytes	gp90 shared with Mac-1; gp180 may be related to interferon ²³ . Antibodies inhibit T cell responses
Relationship to Ig unknown (not all antigens listed)						
CD2	gp50	T11, LFA-2	NA	NA	Thymocytes, T lymphocytes	Receptor for sheep erythrocytes. Antibodies can be mitogenic ²⁴
CD5	gp67	T1; Leu 1	Ly-1	OX-19	Thymocytes, T lymphocytes, B-lymphocyte subset	Antibodies augment T-lymphocyte responses

Names for antigen molecules are traditional or, where possible, the new nomenclature for human antigens is used (see ref. 14). Many of the data are described in ref. 14 (man), refs. 7 and 15 (mouse) and ref. 16 (rat). Equivalent human, mouse and rat antigens are grouped where the similarities seem strong but sequence data will be needed ultimately to prove species equivalence.

NA, no antibody available (but at the DNA level human OX-2 has been identified (M. J. Clark, G. McCaughan and A. N. Barclay, unpublished observations) as has mouse CD3 (ref. 17)). gp, Glycoprotein; p, protein; numbers give the approximate relative molecular mass (M_r) $\times 10^{-3}$.

the evolutionary starting point for Ig was a single domain which gave rise to a structure like an Ig light-chain from which all the Ig chains evolved. This concept holds good for all the molecules in the figure and it seems likely that the primordial molecules were involved in cell surface functions. It is particularly interesting that the functions of the Ig-related molecules include interactions between members of the Ig superfamily¹² (for example, Tcr and MHC; Poly Ig R and Ig A or Ig M; CD8 and MHC class I?; CD4 and MHC class II?). Such reactions may be the general rule, which suggests that the starting point was a recognition system in which Ig-like domains on one cell interacted with identical structures on another. Gene duplication and divergence could have led to interactions based on pairs of dissimilar Ig-related molecules and to the systems that now function in vertebrates.

Since the lymphocyte-based immune system has not been convincingly shown in invertebrates, it is probable that Ig-like structures first evolved on cells that had no immune function. Primitive sensory cells may have been the starting point since vertebrate neurones express Thy-1 and MRC OX-2 antigens and these seem the best candidates for contemporary structures corresponding to the postulated one- and two-domain primordial molecules. It could be argued that the CD8 structure is close to the primordial domain, except that it is most closely related in sequence to light-chain V domains rather than being apparently equidistant from the various V-like structures, as is Thy-1 antigen. Also CD8 and V-kappa genes are closely genetically linked¹³ in man and mouse suggesting that they had a common immediate precursor, possibly with a two-domain structure. □

- Littman, D.R., Thomas, Y., Maddon, P.J., Chess, L. & Axel, R. *Cell* **40**, 237 (1985).
- Sukhatme, V.P., Sizer, K.C., Vollmer, A.C., Hunkapiller, T. & Parnes, J.R. *Cell* **40**, 591 (1985).
- Clark, M.J., Gagnon, J., Williams, A.F. & Barclay, A.N. *EMBO J.* **4**, 113 (1985).
- Boyse, E.A. & Old, L.J. *A. Rev. Genet.* **3**, 269 (1969).
- Immun. Rev.* **74** (1983).
- Reinherz, E.L., Meuer, S.C. & Schlossman, S.F. *Immun. Rev.* **74**, 83 (1983).
- Walker, I.D. *et al. Immun. Rev.* **82**, 47 (1984).
- Thomas, M.L. & Green, J.R. *Eur. J. Immun.* **13**, 855 (1983).
- Snow, P.M. *et al. J. Immun.* **133**, 2058 (1984).
- Kavathas, P. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 7688 (1984).
- Hill, R.L. *et al. Proc. natn. Acad. Sci. U.S.A.* **56**, 1762 (1966).
- Williams, A.F. *Nature News and Views* **308**, 12 (1984).
- Sukhatme, V.P. *et al. J. exp. med.* **161**, 429 (1985).
- Bernard, A. *et al. Leucocyte Typing* (Springer, 1984).
- Immun. Rev.* **68** (1982).
- Mason, D.W. *et al. Immun. Rev.* **74**, 57 (1983).
- van der Eijzen, P. *et al. Nature* **312**, 413 (1984).
- Leifer, D. *et al. Science* **224**, 303 (1984).
- Webb, M. & Barclay, A.N. *J. Neurochem.* **43**, 1061 (1984).
- Littman, D.R. *et al. ICSU Short Rep.* **2**, 233 (1985).
- Thomas, M.L. *et al. Cell* (in the press).
- Remold-O'Donnell, E. *et al. J. exp. Med.* **159**, 1705 (1984).
- Springer, T.A., Teplow, D.B. & Dreyer, W.J. *Nature* **314**, 540 (1985).
- Meuer, S.C. *et al. Cell* **36**, 897 (1984).
- Williams, A.F. & Gagnon, J. *Science* **216**, 696 (1982).
- Chien, Y. *et al. Nature* **312**, 31 (1984).
- Kimball, E.S. & Coligan, J.E. *Contemp. Topics molec. Immun.* **9**, 1 (1983).

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Plant morphogenesis

Oligosaccharide floral messages?

from Keith Roberts

ON page 615 of this issue, Peter Albersheim and colleagues present some initial results that clearly suggest new explanatory hypotheses for the normal growth and development of plants. Their general suggestion is that locked up in the bewildering array of very complex polysaccharides that form the plant cell wall there are short oligosaccharide sequences which, when released, form a set of important signalling molecules that plant cells can use in a variety of ways¹.

There are fundamental differences between the ways that plants and animals develop, most of which rest on the fact that plant cells are immobilized inside a relatively rigid cell wall. Since the wall precludes cell movement, orderly morphogenesis of plants and their organs must depend crucially on a combination of precisely positioned planes of cell division, and controlled and coordinated cell growth². It is a salutary fact that we know very little either about the nature of the spatial controls, or about the molecular mechanisms involved in the determination and control of morphogenetic events. This great area of relative ignorance is all the more surprising when one considers that the plant kingdom offers wonderful experimental systems with unparalleled plasticity of developmental programmes, and that intensive research on plant growth regulators has been going on for over half-a-century.

The whole field is clearly in need of new models that can stimulate fresh approaches to the problem, and the suggestion that oligosaccharides are regulatory molecules provides a good start. The particular experiments reported on page 615, which demonstrate that artificially produced cell wall fragments can switch the experimentally induced pathway of morphogenesis in a model plant system, should be seen in the context of the more general hypothesis.

In the course of chemical work describing the variety of polysaccharides in the primary cell wall, Albersheim and his group found that oligosaccharides released in very low concentrations from the wall of the fungal pathogen *Phytophthora* triggers the expression of plant genes in a response that leads the plant to produce an antibiotic, or phytoalexin^{3,4}. (Oligosaccharides that induce plants to produce phytoalexins are called elicitors and their role in stress and pathogenicity has been discussed recently in these columns⁵.) It was later found that fragments of the plant cell wall, or endogenous elicitors, might also be involved in the response. This fact, linked with the general realization that signalling molecules in plants are probably small water-soluble molecules that seem not to include the pep-

tide and amino acid derivatives used in animal cell signalling, led to the idea that cell wall oligosaccharides might be used more generally by plants as signalling ligands.

Albersheim coined the term oligosaccharins for regulatory molecules that are derived from plant cell wall polymers. Those that have been described so far are 7-15 sugar residues long and have all been obtained either by chemical or enzymatic degradation of the pectic or hemicellulosic fractions of the cell wall. The range of biological effects that oligosaccharins can elicit is wide and includes the inhibition of auxin-induced growth in peas and the inhibition of flowering in *Lemna*⁶.



Scanning electron micrograph of flowers on a tobacco explant, each with an ovary surrounded by stamens. (Courtesy L. Jehanno and K. Tran Thanh Van.)

The new data, which considerably extend the 'oligosaccharin hypothesis' to include a possible regulatory role for oligosaccharins in normal organogenesis, come from a collaboration between Albersheim's group and that of K. Tran Thanh Van. In her elegant system of controlled morphogenesis in thin cell-layer explants of tobacco, controlled combinations of auxin, cytokinin, and pH are used to dissect the regeneration of roots, vegetative buds, floral buds or callus on the explants⁷. In the present experiments sets of oligosaccharides,

one prepared chemically and the other enzymatically from cell-wall pectin, are shown to switch the expected pathway of morphogenesis at concentrations estimated to be no more than 10^{-8} - 10^{-9} M. Thus, with one of the preparations, flower buds can be switched to vegetative buds at pH 3.8, but at pH 6.0 the switch is in the opposite direction. With the other preparation, flowers give way to vegetative buds at pH 3.8, roots are induced instead of only callus at pH 5.0, and at pH 6.0 calus and vegetative buds become callus and roots.

Although neither the chemical identity of the oligosaccharins involved nor any hints as to their mode of action are yet known, the effects themselves are very dramatic and immediately pose exciting questions about the possible role of the oligosaccharins *in vivo* and in the practical context of plant regeneration. The area of work in plant development opened up by oligosaccharin probes is enormous, and already collaborations are in hand to examine the changes in gene expression induced by them in this particular system.

What these experiments throw into very clear focus is our ignorance of the molecular events involved in cell signalling and recognition mechanisms in plants. As yet, know next to nothing about cell surface receptors or signal transduction. The tobacco explant system, with its interaction of hormones, sugar and pH, may be too complex for comfort, but the oligosaccharin hypothesis certainly opens the way to a molecular understanding not only of plant morphogenesis but also of plant cell receptors and signalling mechanisms in general. □

1. Tran Thanh Van, K. *et al.* *Nature* **314**, 615 (1985).
2. *The Cell Surface in Plant Growth and Development* (ed. Roberts, K. *et al.*) *J. Cell Sci.* suppl. 1 (in the press).
3. Darvill, A.G. & Albersheim, P. *Ann. Rev. Plant Physiol.* **35**, 243 (1984).
4. McNeil, M. *et al.* *Ann. Rev. Biochem.* **53**, 625 (1984).
5. Cullis, C. *Nature News and Views* **310**, 366 (1984).
6. Darvill, A.G. *et al.* in *The Cell Surface in Plant Growth and Development* (ed. Roberts, K. *et al.*) *J. Cell Sci.* suppl. 1 (in the press).
7. Tran Thanh Van, K. *Ann. rev. Plant Physiol.* **32**, 291 (1981).

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Early Americans

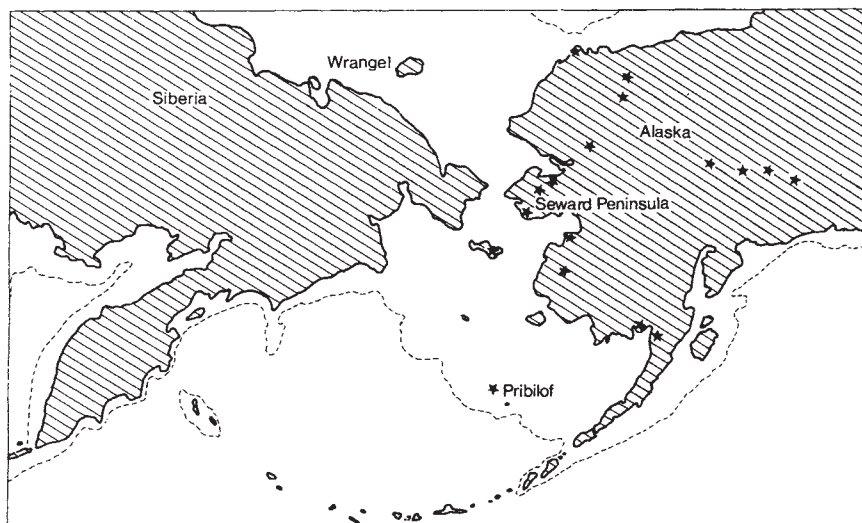
Land bridge of Duvanny Yar

from Paul A. Colinvaux

PRESENT uncertainties about the history of the Bering land bridge and hence of the first peopling of the Americas seem close to resolution. The debate among Siberian geologists is being settled in favour of the modern interpretation, closely associated with Academician N.A. Shilo, who visited Alaska last summer and discussed with a US team what studies would be necessary in Siberia and Alaska to resolve many of the remaining uncertainties.

In the last glaciation, the Bering land

bridge fused Siberia to Alaska as an ice-free plain of continental proportions (see figure). Archaeologists who claim that people lived in America more than 20,000 years ago require the land bridge to have been inhabited early in glacial times; those who dismiss all evidence for pre-Clovis peoples in the New World are content for the first inhabitants of the land bridge to have been in place only in the late glacial, shortly before melting glaciers flooded their new homeland. Either way, the inhabitants orig-



Map of the Bering Strait study area. The broken line indicates the Bering land bridge and asterisks mark the sites of the pollen analyses that have been made. Will they be matched in Siberia?

inates in Siberia, where people are known to have lived as hunters at the forest steppe ecotone over thirty thousand years ago.

The contribution of Shilo and his followers has been to define the geomorphology of north-east Siberia so explicitly that firm environmental limits for the land bridge in full glacial times are established. In eastern Siberia, large rivers flow north and are separated by interfluvies that are hundreds of kilometres wide. The surface of the interfluvies has a topography of flat hillocks and depressions called *yedoma* and *alasy*. It has been evident for some time that the *alasy* depressions are thermokarst features melted out of a once uniform plain of *yedoma* silt.

For many years, Siberian geologists identified the *yedoma* deposits as alluvium and suggested that there had been huge shallow lakes in which the silt settled. Shilo came to question this view during his many years in Siberia, working on gold placers (as a geomorphologist and as a student of frozen mammoth carcasses), before he became head of the Far-Eastern Science Research Centre in Vladivostok¹. He argued that the *yedoma* silt was not alluvium, but loess. This hypothesis has been further developed by Boris Yurtsev² and S.V. Tomirdiaro³, who have marshalled the data of

palaeobotany, mineralogy and geomorphology until the argument for a loessic origin for the *yedoma* has become compelling. Thus older Russian descriptions of Beringian environments are turned on their heads, with 'dry, cold and windswept' substituted for the 'wet and flooded'; it now seems that the silt was blown over an arctic landscape and frozen into place. The dry, cold time of the loess accompanied the Duvanny Yar (Würm-Wisconsin) glaciation in Siberia. The cutting of *alasy* depressions by thermokarst is now dated, by radiocarbon, to the Holocene.

D.M. Hopkins, the US authority on the Pleistocene geology of Bering Strait⁴, who travelled to some of the Siberian sites with Yurtsev in 1981, agrees with the interpretation of the Shilo school. The *yedoma* is comparable to the 'muck' of the Yukon gold placers, and to the thick loess mantle on the central Seward Peninsula⁵. Hopkins also finds evidence for sand, as well as loess, blowing across Alaska, from deep in the interior down to the south land-bridge coast at the Pribilof Islands (see figure).

This view of the land bridge of Duvanny Yar times as dry, cold and barren has also been developed independently from the evidence of pollen in lake sediments. Pollen spectra dated to the land bridge on the Alaskan side represent a tundra that has no exact modern analogue and probably had a less complete vegetation cover than does modern Alaska. The extreme view is that of Ritchie and Cwynar⁶, who use the first good pollen influx data to argue that the land-bridge tundra was as sparse as polar desert. Other pollen analysts argue for something like a dry facies of modern tundras but with fewer sedge meadows and almost devoid of dwarf shrubs^{7,8}. In any case, pollen data seem quite incompatible with a more productive vegetation, like steppe or grassland. When Yurtsev talks of the 'tundra-steppe' that covered the *yedoma* as it formed, he likens this to the sparse tundra of modern Wrangel Island in the Arctic Ocean, his ideas thus converging with the conclusions of pollen analysts

on the Alaskan side².

This reconstruction is in direct conflict with the hypothesis that the Duvanny Yar land bridge supported a complex biocoenosis of big game animals and, hence, a considerable production of plants — the so-called 'mammoth steppe'^{8,10}. Archaeologists who argue for early peopling of the New World rely on the availability of considerable populations of mammoths and other game as the resource of their first Americans^{11,12}. If the Shilo school and the Alaskan pollen analysts interpret the Duvanny Yar ecosystem correctly, it is difficult to see how the necessary population of big game could have been supported. In this event, interest would turn to the late glacial land bridge, when changing climate could have made the southern land-bridge coast more hospitable, just in time for the immediate ancestors of the Clovis culture to supply the first Americans.

As accelerator-dating increasingly brings into question claims of early American sites of human habitation¹³, the idea of a frigid barrier of a land bridge, more than 14,000 years ago, becomes even more appealing. But obviously this reconstruction requires further testing and an important test will be found in applying pollen analysis to lake sediments of the Siberian side. With that in mind, the Climate Dynamics and Polar Earth Sciences programmes of the US National Science Foundation have provided funds for a team from the University of Washington and Ohio State University to work with Academician Shilo and his group on raising sediment cores from a series of lakes in Soviet Beringia. The US team will take their rubber boats and Livingstone sampling equipment, closely modelled on the equipment first introduced into palaeoecology for use in Alaskan studies¹⁴. The two teams plan to use this equipment in Siberia later this year to raise duplicate cores from each lake, and thereafter to conduct pollen and other palaeoecological analyses in parallel.

East and West are divided at the Bering Strait, but we propose to standardize research methods for a final reconstruction of the years when the Strait was abolished and the continents were fused. □

100 Years Ago Far-sightedness

IN THE spring of 1837 I was travelling from Rome, northwards, by 'Vetturino,' and from the summit of Apennine on the road between Florence and Bologna, I saw, with astonishment, the whole range of the Swiss Alps, not merely distinguishable but conspicuous. Measured on the map in a direct line the nearest part of the range was distant about 200 miles. The extreme portions, including Mont Blanc, were considerably more. I have no doubt that the atmospheric conditions were unusually favourable. As a peasant, living on the spot, shortly passed, I asked him what mountains they were — to which he immediately answered to my surprise, that they were the mountains of Switzerland.

J. HIPPLEY

From *Nature* 31 553, 16 April 1885.

1. Shilo, N.A. *Tr. Inst. Magadan Akad. Nauk SSSR* 11, 154 (1964).
2. Yurtsev, B.A. *Paleoecology of Beringia* (ed. Hopkins, D.M. et al.) 157 (Academic, 1982).
3. Tomirdiaro, S.V. *Paleoecology of Beringia* (ed. Hopkins, D.M. et al. (Academic, 1982).
4. Hopkins, D.M. *Science* 129, 1519 (1959).
5. Hopkins, D.M. *Paleoecology of Beringia* (ed. Hopkins, D.M. et al.) 1 (Academic, 1982).
6. Ritchie, J.C. & Cwynar, L.C. *Paleoecology of Beringia* (ed. Hopkins, D.M. et al.) 113 (Academic, 1982).
7. Ager, T.A. *Paleoecology of Beringia* (ed. Hopkins, D.M. et al.) 75 (Academic, 1982).
8. Colinvaux, P. & West, F. *Q. Rev. Archaeol.* 5, 10 (1984).
9. Matthews, J.V. *Am. Quat. Ass. Abstracts* 4, 73 (1976).
10. Guthrie, R.D. *Paleoecology of Beringia* (ed. Hopkins, D.M. et al.) 307 (Academic, 1982).
11. Morlan, R.E. *Archaeol. Survey Canada Pap.* No. 94 (1980).
12. West, F.H. *Q. Rev. Archaeol.* 4, 13 (1982).
13. Bada, J.L. et al. *Nature* 312, 442 (1984).
14. Livingstone, D.A. *Ecology* 36, 137 (1955).

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Molecular biology

Reverse transcriptase rides again

from Harold E. Varmus

JUST fifteen years ago an explosive growth in RNA tumor virology was triggered by the discovery of reverse transcriptase^{1,2} — the enzyme of retroviruses that copies their RNA genome into DNA, which can then become integrated as a 'provirus' into the DNA genome of infected cells. Yet reverse transcriptase in recent years seems to have given up centre stage to oncogenes and proto-oncogenes. If so, there is now furious activity in the wings: recent findings about the *pol* genes of retroviruses — and about related genes in other viruses and in transposable elements of flies and yeast — signal a new era of discovery likely to influence such diverse topics as mechanisms of transposition, molecular evolution, and translational fidelity.

Pol coding domains

What has come to be called a retroviral *pol* gene consists of a large open reading frame, situated between reading frames for core (*gag*) and envelope (*env*) polyproteins, that encodes a protein with both reverse transcriptase RNA-directed DNA polymerase and ribonuclease H activities³ (see the figure). Most retroviruses express *pol* by first synthesizing a *gag-pol* polyprotein (discussed further below) that is subsequently cleaved to mature components, including a virus-associated DNA endonuclease^{4,6}, most likely by a virus-encoded site-specific protease^{7,9}.

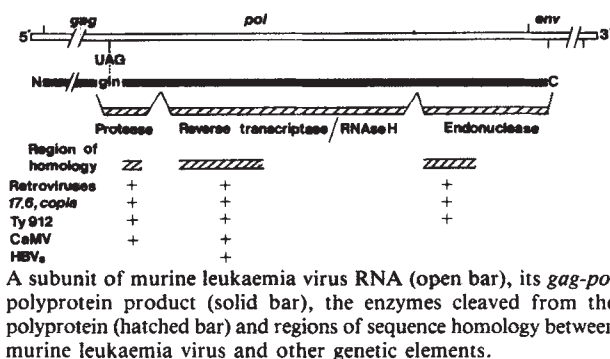
Structural studies of the DNA endonuclease present in avian leukosis and sarcoma viruses (ASLVs) suggested its derivation from the 3' end of *pol*^{4,10}, a suggestion that has since been extended to the endonucleases of other viruses (see below). The source of the protease has been more enigmatic: though associated with the carboxyterminal product of the ASLV *gag* polyprotein⁴, protease activity has not been detected in murine leukemia virus (MLV) *gag* proteins¹¹. Analysis of a *pol* frameshift mutant competent to process its *gag* polyposition and comparison of the amino-terminal sequence of MLV reverse transcriptase with the nucleotide sequence of *pol*¹² suggests that there is room in the region of *pol* immediately downstream of *gag* to encode the protease isolated from MLV particles.

Strong confirmation of these predictions is now in hand. A partial amino acid sequence of a protease purified from MLV corresponds to the sequence predicted from the 5' end of *pol*¹³ (also see below); and a short in-frame deletion mutation, engineered in the same region, cancels processing of both *gag* and *gag-pol* precursors¹⁴.

(The mutant appears to assemble virus particles with normal efficiency and, surprisingly, to contain normal levels of *in vitro* reverse transcriptase activity, though unable to synthesize viral DNA upon infection.)

Site-directed mutagenesis has also been used to document the importance of the putative endonuclease coding domain at the 3' end of the *pol* genes of MLV^{15,16}, ASLV¹⁷, and spleen necrosis virus (SNV)¹⁸. Both deletion and point mutations in this region of cloned MLV and SNV DNA produce virus particles that synthesize apparently normal linear and closed circular DNA intermediates after infection, but fail to integrate viral DNA with the efficiency and precision characteristic of wild type virus^{15,16,18}.

What are the biological roles of the endonuclease? At least two recombinatorial functions can be envisioned. First it may be responsible for a staggered cutting of chromosomal DNA (ostensibly in a sequence-independent manner) to yield the virus-specified four, five, or six base-pair duplications of host sequence that are found to flank proviral DNA. Second, the endonuclease may carry out sequence-specified tailoring of viral DNA so that



proviruses are uniformly joined to host DNA at sites two base pairs from the ends of viral long terminal repeats (LTRs), within the inverted repeats that conclude the LTRs¹⁹. There are now strong reasons to believe that during infection and integration the endonuclease recognizes and is to cut circle junctions — the palindromic sequences formed when the inverted repeats at the end of unintegrated linear DNA are joined to yield circular DNA species. Experimental insertion of a small circle junction fragment into an SNV vector creates a second integration site within the viral genome²⁰ and integrity of the inverted repeats is mandatory for integration²¹. In addition, when an ASLV endonuclease is incubated with single-stranded viral DNA *in vitro*, it prefers to cut close to the expected sites²².

The claim that hepatitis B viruses (HBVs)

and cauliflower mosaic virus (CaMV) replicate through RNA intermediates with the help of reverse transcriptase (see ref. 23) has received striking support from computer searches: Toh *et al.* were first to report a persuasive relatedness between the amino-terminal portion of retroviral reverse transcriptases and products of long unassigned open reading frames of HBVs and CaMV²⁴. Comparisons of products of disparate retroviruses have shown that all three functional domains described for the MLV *pol* gene are strongly conserved, although not always within a single open reading frame (see below)²⁵⁻²⁷.

Similarly successful games have now been played with the newly-determined sequences of the mobile *copia*-like genetic elements of *Drosophila* and the mobile *Ty* elements of *Saccharomyces*. These transposons had previously been recognized to resemble retroviral proviruses from their general organization and some limited sequence similarities within, and adjacent to, their LTRs (reviewed in ref. 28). Saigo, Miyata and their colleagues^{27,29} have since found that protein sequences homologous to retroviral protease, reverse transcriptase, and endonuclease are encoded within the second of three long open reading frames of the *copia*-like element 17.6, where they are arranged similarly to the domains described for MLV *pol*. In *copia* itself, which has only a single, extensive open reading frame, the endonuclease region instead lies between an upstream protease and a downstream reverse transcriptase region³⁰. The yeast element, *Ty* 912, has a *copia*-like arrangement of domains within the second of two open reading frames³¹.

Transposition via RNA

There is, at least, superficial congruence between the sequence relationships listed in the figure and the evidence available about their predicted functional consequences. Thus, for HBVs and CaMV, which contain reverse

transcriptases but not endonucleases, the replication cycle seems to include RNA-directed DNA synthesis but not orderly or efficient integration into the host genome. Several transposable elements of *Drosophila* and *Saccharomyces* are organized in a fashion consistent with the use of a retrovirus-like integrative mechanism, dependent upon the putative endonuclease domains; but, until recently, evidence for reverse transcription has been limited to the existence of linear and circular DNA forms of *copia*-like elements that resemble unintegrated retroviral species³²⁻³⁴ and virus-like particles associated with *copia* RNA that display incompletely studied reverse transcriptase activity³⁵.

This rather tentative situation has now been dramatically altered by experiments that show beyond reasonable doubt that *Ty* elements, like retroviral proviruses,

transpose via RNA intermediates. For what seems likely to rank among the ten best scientific stories of the year, Boeke *et al.* constructed a molecularly-tagged *Ty* element³⁶, in which the promoter of the 5' LTR is replaced by the inducible *GAL1* promoter, so that *Ty* transpositions to a target yeast plasmid³⁷ could be shown to be dependent on transcriptional activity. Moreover, *Ty* LTRs were shown to be regenerated in each transposition cycle, in the manner of retroviral LTRs such that differences between the LTRs in the original element are eliminated from the newly synthesized copy received by the target plasmid. Finally, in a manoeuvre destined to convince even those unfamiliar with the acrobatics of retroviral replication that an RNA intermediate is used, Boeke *et al.* prove that an intron placed in an innocuous position in the moveable element can be precisely removed during transposition.

In the wake of the *Ty* results, few will question the hypothesis that *copia*-like elements of *Drosophila* and retrovirus-like elements of mammals (for example, VL-30 and intracisternal A particle DNAs³) also transpose and amplify their numbers through RNA intermediates, even though they lack the ability to make extracellular virus particles. But acceptance of this idea carries with it an evolutionary dilemma: are retroviral proviruses of vertebrates, with their capacity to spread as viruses from cell to cell and animal to animal, to be considered as advanced versions of more primitive elements present in all eukaryotes? Or are such elements the remnants of ancient retroviral infections, retaining only in occasional instances the capacity to mediate limited (intracellular) transposition?

Translational suppression

It has been apparent for several years that something peculiar must occur at the boundary of *gag* and *pol* (see figure) to allow synthesis of a *gag-pol* polypeptide, at 1-5 per cent the efficiency of *gag* polypeptide³. From the first definitions of the precursors to MLV and ASLV reverse transcriptases^{38,39}, at least two possibilities have been on the table: either an inefficient splicing event generates a *gag-pol* mRNA that lacks the *gag* terminator, or the terminator must occasionally be suppressed or otherwise bypassed in translation. Those favoring simple termination suppression took heart some years ago from the demonstration by Philipson *et al.*⁴⁰ that MLV virion RNA could be translated *in vitro* to yield both *gag* and *gag-pol* precursors, and that the latter was increased at the expense of the former by addition of yeast amber-suppressor tRNA. In similar experiments with Rous sarcoma virus (RSV) RNA, however, addition of suppressor tRNA provoked the synthesis of a protein only slightly longer than the *gag* precursor⁴¹. Nucleotide sequencing then revealed that *gag* and *pol* are in different, albeit overlapping, reading frames in the RSV

genome⁴², whereas MLV *gag* and *pol* are in the same frame and separated only by an amber codon⁴³, as anticipated from the *in vitro* translation studies.

The translational suppression school thereupon fell into mild disfavour and, since all retroviruses generally solve their problems in similar ways, it was widely assumed that they must make a small amount of atypically spliced *gag-pol* mRNA, despite the absence of recognizable splicing signals. The constitutive synthesis of *gag-pol* protein *in vitro* was ascribed to inadvertent packaging of *gag-pol* mRNA.

Oroszlan and his colleagues¹³ have now turned all eyes back to translational suppression, by showing unambiguously that the amber terminator of the MLV *gag* is suppressed during synthesis of the *gag-pol* polypeptide. Amino-terminal sequencing of the protease of MLV shows it to be a cleavage product of the region of the polypeptide encoded by the *gag-pol* junction. More importantly, the first four amino acids are encoded by the *gag* sequence preceding the amber codon (UAG in figure); those beginning with number six are encoded by the *pol* sequence following the terminator; and the fifth amino acid is glutamine (gln in figure) in at least 80-90 per cent of the protease molecules. What determines that the amber codon is misread as glutamine instead of termination 1-5 per cent of the time? What species of tRNA is involved? Can other genes be affected in this way?

These self-evident questions are accompanied by less obvious, but no less provocative, ones. What should we now make of RSV, in which *gag* and *pol* are out of frame? Is there a mechanism for translational frameshifting that works as regularly upon RSV RNA as amber suppression does upon MLV RNA? Oblique suggestions that this is the case have come from recent

studies of yeast *Ty* elements. The two major open reading frames of *Ty* 912 are overlapping and the second has no initiation codon until well into the open frame; rigorous attempts to discover an appropriately spliced mRNA have failed; and the size of *Ty-lacZ* fusion proteins indicates that translation of the second reading frame initiates from the 5' end of the first³¹. From this and a related study by Mellor *et al.*⁴⁴, it seems likely that translational frameshifting is required for expression of the second frame, which is encoded proteins related to the products of retroviral *pol* genes.

Cursory examination of the relationships between the first (*gag*-like) and second (*pol*-like) coding domains of a number of retrovirus-related elements shows a tantalizing potential for diversity in the synthesis of *pol* proteins and their homologues. In *Ty* 912, the second frame is '+1' with respect to the first³¹, whereas the RSV *pol* frame is '-1' with respect to *gag*⁴², and the same is true for the *Drosophila* element^{17,629}. On the other hand, *copia* seems to be similar to MLV, with *gag*- and *pol*-like sequences in the same frame, but not separated by a termination codon³⁰. The frequency of termination codons between the *gag* and reverse transcriptase coding regions of HTLV-I⁴⁵ and II⁴⁶ and bovine leukaemia virus^{47,48} demands either splicing or multiple suppression events for expression of *pol*; however, translation of sequences that begin near the 3' end of *gag* in the '-1' frame would yield proteins related to retroviral proteases^{27,49}.

In sum, an astonishing amount has been learned recently about functional and structural similarities among the *pol* genes of retroviruses and related elements — a good augury for the next fifteen years of reverse transcription. □

1. Temin, H.M. & Mizutani, S. *Nature* **226**, 1211 (1970).
2. Baltimore, D. *Nature* **226**, 1209 (1970).
3. Weiss, R., Teich, N., Varmus, H.E. & Coffin, J. (eds) *Molecular Biology of Tumour Viruses: RNA Tumor Viruses* (Cold Spring Harbor, New York, 1982).
4. Grandgenett, D.P., Vora, A.C. & Schiff, R.D. *Virology* **89**, 119 (1978).
5. Kopchick, J.J. *et al.* *J. Virol.* **37**, 274 (1981).
6. Nissen-Meyer, J. & Ness, I. *Nucl. Acids Res.* **8**, 5043 (1980).
7. von der Helm, K. *Proc. natn. Acad. Sci. U.S.A.* **74**, 911 (1977).
8. Dittmar, K.J. & Moelling, K. *J. Virol.* **28**, 106 (1978).
9. Yoshinaka, Y. & Luftig, R.B. *Cell* **12**, 709 (1977).
10. Grandgenett, D.P., Kraus, R.J. & Hippenmeyer, P.J. *Virology* **130**, 257 (1983).
11. Levin, J.G. *et al.* *J. Virol.* **51**, 470 (1984).
12. Copeland, T.D. *et al.* *Virology* (in the press).
13. Yoshinaka, Y., Katoh, I., Copeland, T.D. & Oroszlan, S. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1618 (1985).
14. Crawford, S. & Goff, S. *J. Virol.* **53**, 899 (1985).
15. Schwartzberg, D. *et al.* *Cell* **37**, 1043 (1984).
16. Donehower, L.A. & Varmus, H.E. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6461 (1984).
17. Hippenmeyer, P. & Grandgenett, D. *Virology* **137**, 358 (1984).
18. Panganiban, A.T. & Temin, H.M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7885 (1984).
19. Varmus, H.E. *Science* **216**, 812 (1982).
20. Panganiban, A.T. & Temin, H.M. *Nature* **306**, 155 (1983).
21. Panganiban, A.T. & Temin, H.M. *Cell* **36**, 673 (1984).
22. Duyk, G., Leiss, J., Longiaru, M. & Skalka, A.M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6745 (1983).
23. Varmus, H.E. *Nature News and Views* **304**, 116 (1983).
24. Toh, H. *et al.* *Nature* **305**, 829 (1983).
25. Patarca, R. & Haseltine, W.A. *Nature* **309**, 728 (1984).
26. Chiu, I.M. *et al.* *Science* **223**, 364 (1984).
27. Toh, H. *et al.* *EMBO J.* (in the press).
28. Varmus, H.E. In *Mobile Genetic Elements* (ed. Shapiro, J.) (Academic Press, New York, 1983).
29. Saigo, K. *et al.* *Nature* **312**, 659 (1984).
30. Mount, S. & Rubin, G. *Mol. cell. Biol.* (in the press).
31. Clare, J. & Farabaugh, P. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
32. Flavell, A.J. & Ish-Horowitz, D. *Nature* **292**, 591 (1981); *Cell* **34**, 415 (1983).
33. Flavell, A.J. *Nature* **310**, 514 (1984).
34. Mossie, K.G., Young, M.W. & Varmus, H.E. *J. mol. Biol.* (in the press).
35. Shiba, T. & Saigo, K. *Nature* **302**, 119 (1983).
36. Boeke, J.D., Garfinkel, D.J., Styles, C.A. & Fink, G.R. *Cell* **40**, 491 (1985).
37. Scherer, S., Mann, C. & Davis, R.W. *Nature* **298**, 815 (1983).
38. Jamjoom, G.A., Naso, R.B. & Arlinghaus, R.B. *J. Virol.* **78**, 311 (1977).
39. Opperman, H., Bishop, J.M., Varmus, H.E. & Levintow, L. *Cell* **12**, 993 (1977).
40. Philipson, L., Andersson, P., Olshevsky, U., Weinberg, R., Baltimore, D. & Gesteland, R. *Cell* **13**, 189 (1978).
41. Weiss, S. *et al.* *Cell* **15**, 607 (1978).
42. Schwartz, D., Tizzard, R. & Gilbert, W. *Cell* **32**, 853 (1983).
43. Shinnick, T.M., Lerner, R.A. & Sutcliffe, J.G. *Nature* **293**, 543 (1981).
44. Mellor, J. *et al.* *Nature* **313**, 293 (1985).
45. Seiki, M., Hattori, S., Hirayama, Y. & Yoshida, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3618 (1983).
46. Shimotohno, K. *et al.* *Proc. natn. Acad. Sci. U.S.A.* (in the press).
47. Sagata, N. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **82**, 677 (1985).
48. Rice, N. *et al.* *Virology* (in the press).
49. Sagata, N. *et al.* *FEBS Lett.* **178**, 78 (1984).

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Novel DNA sequence representations

SIR — Recent contributions from Lathe and Findlay¹ and Hayashi and Munkata² are manifestations of a widespread concern that the conventional (letter-sequence) representations of long DNA sequences are unsuitable for purposes other than elementary information storage. With regard to comprehensibility, such data appear to be analogous to the atomic coordinate tables seen in the specialized journals of X-ray crystallographers. Data in such form cannot be remembered, compared to other similar data, or scanned for certain features without some computer manipulations.

Lathe and Findlay's suggested sequence representation is based on four vertical lines of unequal lengths, each assigned to one of the four nucleotide bases¹. (This idea appears to be related to the more general computer-based approach used by Daniels *et al.*³.) For relatively short sequences the method creates attractive mnemonics capable of reflecting certain symmetry features of the sequence. However, for long DNA sequences of several thousand nucleotides this method would be limited by the same problems as those of the letter-sequence method — a compromise between excessive figure size and legibility, lack of indication of global sequence characteristics, and the impossibility of resolution adjustment. The conventional letter-sequence method seems adequate for short DNA where the conveyance of the short-range details of the information is the most important factor. Thus, the suitability of any alternative method must be tested not only on short but also on long DNA sequences.

We have proposed a graphic representation method⁴⁻⁶ based on the idea of mapping the information content of a DNA sequence into a three-dimensional space curve such that the shape of the curve represents the sequence of nucleotides. A

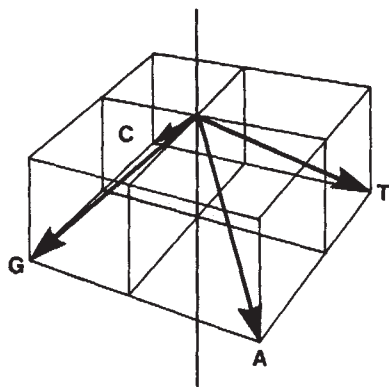


Fig. 1 Perspective illustration of the four small elemental space vectors used to depict the four nucleotides in the H-curve method of DNA sequence representation. The H curve for a DNA sequence is constructed by a head-to-tail assembly of these vectors according to the nucleotide sequence of the DNA.

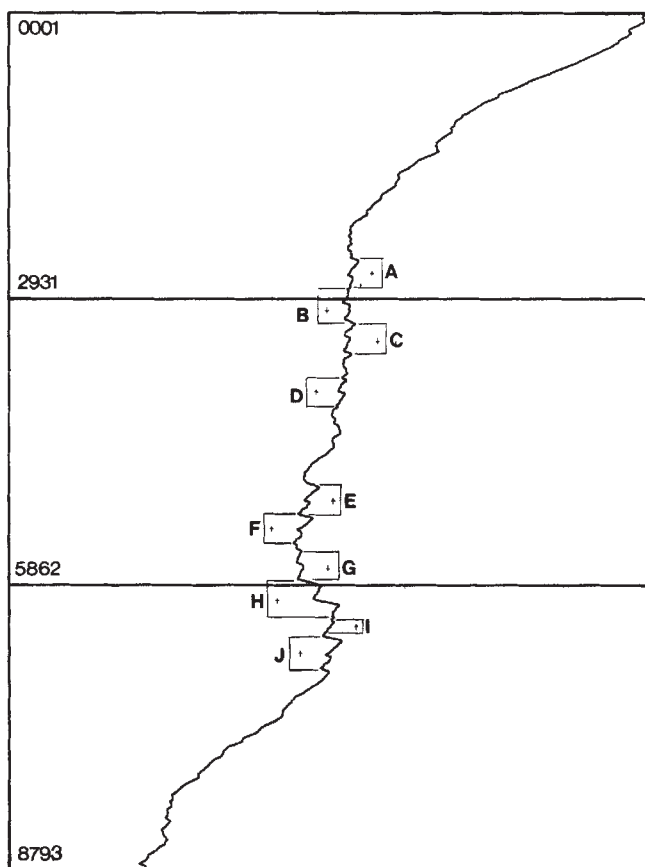


Fig. 2 The H curve of an 8,793-nucleotide-long human DNA sequence (an expressed isotype (5β) of the β -tubulin gene cluster⁸). The projection of the curve shown is the front view with the elemental vectors (here unresolvable) for the G and C nucleotides pointing to the left and those for the A and T nucleotides to the right. The vertical central portion of the curve corresponds to an unusual intron containing a cluster of ten 300-nucleotide-long *Alu* sequences (sections marked with letters). The top of the curve is the 5' end. The vertical reference lines are 2,931 nucleotides apart and the smoothing factor⁵ (w) was 10.

short space vector of characteristic direction is assigned to each of the four nucleotides (Fig. 1) and the curve is assembled from such vectors by joining them head-to-tail in the order of the nucleotides in the DNA sequence. The front and the side views of these 'H-curves' (or their stereoscopically viewed three-dimensional version) can provide not only short-range detail but can also reflect global nucleotide-distribution trends. For instance, DNA regions rich in certain nucleotides will result in H-curve regions shifting in characteristic directions. An important property of these curves is that they can be generated or viewed at different degrees of resolution. At high resolution the individual nucleotides of the sequence are observable but at lower resolution, (when curves are condensed to a relatively short vertical length) the regional variations of nucleotide-distribution patterns along the DNA sequence are emphasized. This latter property of our curves makes them particularly suitable for representing very long DNA sequences. (We recently published the evaluation of the H-curve of the λ genome sequence of 48,502 nucleotides⁷.)

The curve shown in Fig. 2 illustrates the utility of our representation method for the display and location of character-

istic repeating elements on a lengthy eukaryotic DNA sequence.

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1. Lathe, R. & Findlay, R. *Nature* 311, 610 (1984).
2. Hayashi, K. & Munkata, N. *Nature* 310, 96 (1984).
3. Davidson, N. & Szybalski, W. in *The Bacteriophage Lambda* (ed. Hershey, A. D.) 62-70 (Cold Spring Harbor Laboratory, New York, 1971).
4. Hamori, E. *Fedn Proc.* 40, 1647 (1981).
5. Hamori, E. & Ruskin, J. *J. biol. Chem.* 258, 1318-1327 (1983).
6. Hamori, E. *Fedn Proc.* 42, 2263 (1983).
7. Hamori, E. *Gene analyt. Techq.* 42, 2263 (1983).
8. Lee, M. G.-S., Loomis, C. & Cowan, N. J. *Nucleic Acids Res.* 12, 5823-5836 (1984).

TATHE AND FINDLAY REPLY — Sequences of the four letters G, A, T(U) and C, are particularly intractable to visual analysis. An ideal format would (1) reveal local features such as recognition sites for enzymes interacting with nucleic acids; (2) reveal large-scale base distribution patterns, and (3) be machine-readable. It is improbable that a single method can meet all three requirements.

The methods proposed by Hamori¹ and ourselves² are quite different. We are nevertheless struck by the parallels between the two formats (angle-vector H-curve and line-extension) and the two methods of

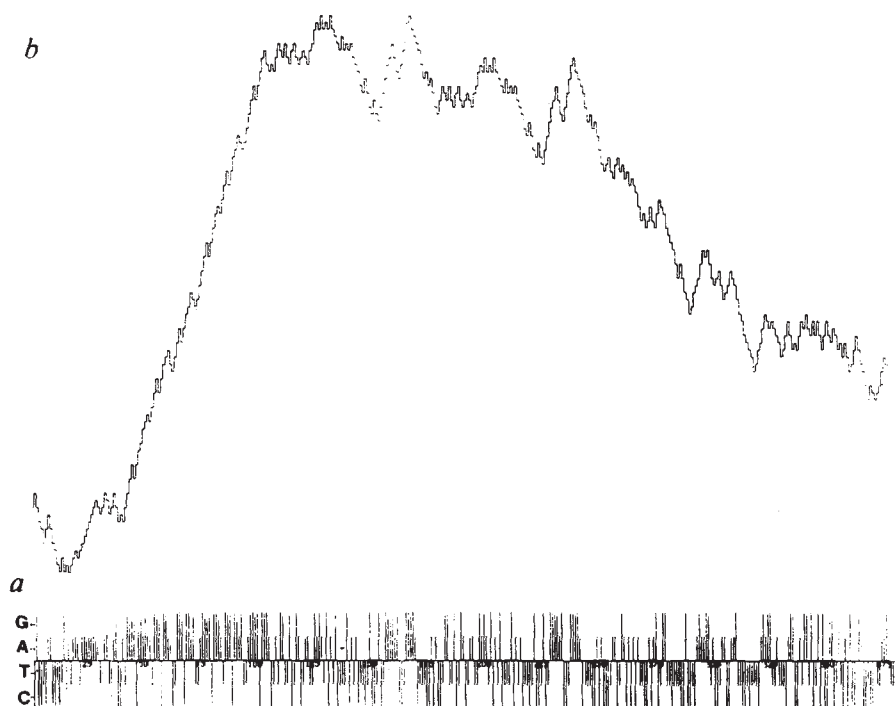


Fig. 1. The first 376 nucleotides of the SV40 genome displayed by the standard (a) and cumulative (b) line-extension formats. In both diagrams the four nucleotides G, A, T and C are given y-axis values of +2, +1, -1 and -2, although the scale of b has been reduced in this dimension. The direct 72-base pair repeat is clearly visible (region 100-250). Restriction recognition sequences form (usually) rotationally-symmetrical patterns in a and symmetrical peaks or troughs in b.

resolving oligonucleotides for nucleic acid sequencing — wandering spot³ (Hamori) and thin-layer acrylamide gel electrophoresis⁴ (our method). This superficial coincidence may reflect a limited number of possible formats.

Our first concern has been to devise a method of transferring printed sequences, using a scanning light-pen, from the printed page to an information retrieval system. Simple variants of bar-thickness identification codes were ruled out since deciphering is particularly taxing. Angle-vector methods of display such as H-curves are not inherently machine-readable although they reveal, as do plots of G/C content, features of the overall sequence profile. The base-composition curve is, to a close approximation, the first derivative of the (projected) H-curve, and as such embodies the same information. We have also examined formats which reflect the chemical structures of the four bases and which might, by comparison of non-identical recognition sequences, reveal nucleic acid-protein contact points. However, such formats were particularly difficult to interpret by eye.

We have subsequently examined a display which combines features of both the line-extension and angle-vector formats. This cumulative line extension format (which respects the extension ratios for the four bases, Fig.1) gives similar information to that yielded by angle-vector diagrams but is less ambiguous than projected H-curves. Restriction recognition sequences appear here as symmetrical peaks. However, the format is not machine-readable and we have not

proceeded further with this approach.

The two methods under discussion^{1,2} meet different applications. The method we have proposed reveals local features of a DNA sequence and is limited to some 10,000 nucleotides per standard page. The display is machine-readable. In contrast, H-curves accentuate (as do G/C content plots) global features of a large-scale sequence while concealing the detail. They permit 100,000 or more nucleotides to be displayed per page. To this extent the two methods are non-overlapping and provide a visual representation of a particular nucleic acid sequence.

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1. Hamori, E. *Gene analyt. Techq.* 1, 69, (1984)
2. Lathe, R. & Findlay, R. *Nature* 311, 610 (1984)
3. Sanger, F., Brownlee, G.G. & Barrell, B.G. *J. molec. Biol.* 13, 373 (1965)
4. Sanger, F., Nicklen, S. & Coulson, A.R. *Proc. natn. Acad. Sci. U.S.A.* 74, 5463 (1977)

Powers of ten wrongly expressed

SIR — It is surprising how often authors improperly use powers of 10, in the expression of radioactivity measurements particularly. I often read manuscripts in which the ordinates are labelled as follows: 'Radioactivity (c.p.m. $\times 10^{-3}$)' or simply 'c.p.m. $\times 10^{-3}$ ' with figures ranging from 0 to 10 or so.

Since $a \times b = b \times a$, 'c.p.m. $\times 10^{-3}$ ' actually means '10⁻³ c.p.m.' or 'milli-

counts per min'. This is obviously not what the authors mean. The proper way to label such ordinates is either '10⁻³ $\times$ radioactivity (c.p.m.)' or 'Radioactivity (c.p.m. $\times 10^3$)'.

A physical quantity (see page 5 in ref.1) is the product of a pure number and a unit, that is, a particular physical quantity used as a standard, for example:

Mass = 3 $\times$ grams (or 3 g) (1)
Radioactivity = 5,000 $\times$ c.p.m.
(or 5,000 c.p.m.) (2)

If one divides 5,000 by 10³ in equation (2), then 'radioactivity' should also be divided by 10³ (or multiplied by 10⁻³), or 'c.p.m.' should be multiplied by 10³.

Many authors divide both the number and the unit by 10³, thus dividing only the right-hand side of equation (2) by 10³. This is incorrect.

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1. International Union of Pure and Applied Chemistry. *Manual of Symbols and Terminology for Physicochemical Quantities and Units*. 1979 Edition (Pergamon Press, Oxford 1979).

Mass extinction times and correlations

SIR — Rampino and Stothers¹ make the interesting argument that the correlation they found² between ordered sequences of nine mass extinction times and nine galactic plane crossings ($r = 0.996$) is statistically significant, while the correlation that I found³ between the same ordered extinction times and the first nine prime numbers ($r = 0.986$) is not. Of course, this leaves open the question of the significance of the correlation of the ordered extinction times and the first nine odd numbers ($r = 0.995$).

The question of how to test statistical relations between serial data is a serious and difficult one of long standing, going back at least a century to W. Stanley Jevons' attempts to show a connection between sunspots and the business cycle. I believe its resolution in the present case will probably require more data, and certainly a different analysis, than that given by Rampino and Stothers. All monotone series appear highly correlated, all regular monotone series appear even more so. This artefact aside, and even under the authors' most optimistic assumptions, the statistical evidence is simply too weak to reach any conclusion, due to the large variance (relative to the small sample size) of the times between mass extinction dates. The intriguing connection Rampino and Stothers suggest between these two phenomena may well be a real one; their analysis has not yet proved it to be real.

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1. Rampino, M. R. & Stothers, R. B. *Nature* 313, 159-160 (1985).
2. Rampino, M. R. & Stothers, R. B. *Nature* 308, 709-712 (1984).
3. Stigler, S. M. *Nature* 313, 159 (1985).

Records of Halley's comet on Babylonian tablets

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The late Babylonian texts in the British Museum are shown to contain probable observations of Halley's comet at both its 164 BC and 87 BC apparitions. These texts have important bearing on the orbital motion of the comet in the ancient past.

HALLEY'S comet returns to the inner Solar System every 75–80 years, the precise interval depending appreciably on perturbations in its orbit induced by various planets. The comet is unique in being the only known periodic comet that regularly becomes bright enough to attract the attention of the unaided eye. As a result, no other comet has a comparable history. Since the pioneering work of Halley¹, the orbital motion has been investigated carefully and observations of every apparition as far back as 12 BC have been identified confidently².

Despite this impressive record, the history of Halley's comet before 12 BC is relatively obscure. At this early period, Chinese records, so detailed in later centuries, are comparatively poor, although there are two possible references in 87 BC and 240 BC^{2,3}. Greek and Latin sources mention several comets occurring in ancient times, but scant attention is paid to information such as celestial location and exact date of observation. Identification of P/Halley (the prefix denotes the periodic nature of the comet) among such diverse material is very difficult, if not impossible.

A neglected source of cometary observations is the late Babylonian astronomical texts. These inscribed clay tablets were discovered about a century ago and are now mainly in the British Museum⁴. Here we present for the first time two separate Babylonian records of P/Halley from 164 BC (the only extant records of this apparition in the world) and one from 87 BC. It is hoped that these records will enable a more definite orbit of P/Halley to be traced into the ancient past and a deeper understanding of the evolution of cometary orbits to be made with respect to the recent interest in cometary catastrophism.

Orbital parameters

According to Ho Peng Yoke's⁵ catalogue of oriental records of comets and guest stars, some 500 naked-eye comets have been observed over the past 2,200 years (that is, an average of one comet approximately every five years). On the basis of these statistics, there is a significant probability of another bright comet appearing in the same year as P/Halley itself; in fact several such double events are known to have occurred, most recently in 1910. Thus, it is evident that to investigate the most ancient apparitions of Halley's comet, a highly reliable orbital theory is required or an incorrect identification may well be made. Recently, four independent sets of osculating orbital elements have been derived for P/Halley by Chang⁶, Yeomans and Kiang⁷, Brady⁸ and Landgraf⁹. All the solutions cover more than three millennia and are based on a numerical integration of the equations of motion of the comet backwards in time.

The apparent motion of a comet across the celestial sphere is only weakly dependent on small changes in orbital parameters such as eccentricity, inclination and perihelion distance. The slight differences in these parameters deduced by the various authors can safely be neglected. On the other hand, the relative motion with respect to the Earth is very sensitive to variations in the precise date of perihelion passage (T). Hence a question of prime concern is the reliability of values of T as calculated by the authors of refs 6–9.

Both telescopic and pre-telescopic measurements of the position of P/Halley enable the dates of every perihelion passage back to AD 989 to be determined accurately^{2,3,7}. On only two occasions during this period do observational uncertainties exceed 0.5 day (1.5 days in AD 1222 and 0.8 days in AD 1145).

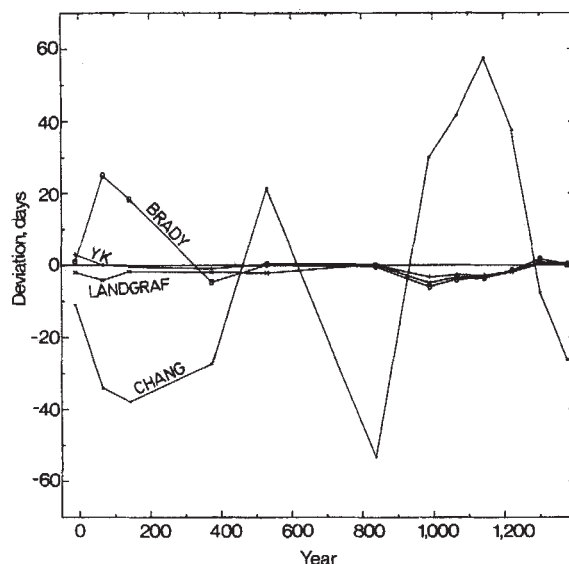


Fig. 1 Plot of the deviations between the calculated and observed dates of perihelion passage for Halley's comet between 12 BC and AD 1378 for the investigations in refs 6–9. YK, Yeomans and Kiang.

Further back in time the precision of observation is generally low but there are several notable exceptions. In AD 837, 530, 374 and 141, careful measurements of the RA of the comet by Chinese astronomers enable values of T to be deduced with uncertainties between 0.1 and 0.5 days. Finally, Chinese observations in AD 66 and 12 BC, although less accurate, still have tolerably small uncertainties of ~2 days.

Over the earlier part of this time range, the results of a comparison between observation and theory for the four investigations cited are summarized in Fig. 1. This diagram emphasizes the high accuracy of Yeomans and Kiang⁷, Brady⁸ and Landgraf⁹ in representing observations as far back as AD 374; discrepancies in T do not exceed ~5 days at any perihelion passage since that date. Similar consistency for the three most ancient well-observed apparitions (AD 141, AD 66 and 12 BC) is obtained in refs 7, 9. However, Brady's⁸ results for T are somewhat discordant in both AD 141 (~20 days error) and AD 66 (~25 days), although the accuracy is again high in 12 BC (discrepancy ~1 day). The relatively low precision of Chang's⁶ solution is evident; errors of a month in T are not unusual.

The mutual discrepancies in the dates of perihelion passage as calculated in refs 6–9 back to 700 BC are shown in Fig. 2. Here, Yeomans and Kiang's data⁷ have been adopted arbitrarily as the standard from which to measure deviations. From Fig. 2 it is apparent that for any apparition of P/Halley back to 87 BC the agreement between refs 7–9 is good; the maximum discord amounts to less than a month. In 164 BC and 240 BC, refs 7 and 9 still tend to support one another, discrepancies being about 15 and 40 days, respectively. However, Brady⁸ deviates from these solutions by some 150 days in both cases. Beyond 240 BC, even the precise years of apparition are in dispute. The apparent lack of useful observational data before 12 BC is a serious handicap to further progress. It is hoped that our discovery

of the Babylonian records for 164 BC and 87 BC will assist in the determination of more accurate solutions in the ancient past.

Babylonian observations

The extant Babylonian cometary observations are recorded in a group of cuneiform texts called *Astronomical Diaries*¹⁰. These texts originally covered the period from ~750 BC to perhaps as late as AD 75, but only a small fraction is preserved; almost all surviving texts are now in the British Museum. The diaries contain day-by-day observations, mainly of the Moon and planets, but also of other phenomena in the sky, some of atmospheric origin¹¹. Comets are mentioned in several of these diaries, the earliest known observation dating from 235 BC. Unfortunately, all contexts are broken, hence it is not possible to give a complete set of observations for the apparition of any comet.

The term used for a comet in the diaries is *šallammu* (or *šallummu*), which in other Babylonian texts can denote a phenomenon similar to a meteor or fireball¹². No etymology of the word is known; even its reading is to some extent uncertain. A meaning 'comet' is suggested by evidence from the diaries which show the *šallammu* to remain in the sky for an extended time (several days or even weeks) so that a meteor explanation is excluded. Although a meteor interpretation cannot be excluded for some of the *šallammu* mentioned in the astrological cuneiform texts, there is no evidence to suggest anything other than a cometary identification when this term is found in the diaries.

Two apparitions of P/Halley seem to be identifiable among the records of comets in the cuneiform diaries. These can be dated confidently as 164 BC and 87 BC. For the earlier of these, two separate records are extant; although both are badly damaged, the two descriptions supplement one another. Unfortunately, only a single brief entry has survived from 87 BC, but this contains several useful details, including a single exact date.

We give below transliterations and translations of each text under the relevant year. The reference numbers preceding each transliteration give the British Museum catalogue number, relevant lines of text and, where appropriate, the location in Sachs's *Late Babylonian Astronomical and Related Texts (LBAT)*⁴. See page 577 for a photograph of BM 41462.

(1) 164 BC

BM 41462: 16f (LBAT 380)

[... šal-la]m-mu-ú šá ina IGI-ma ina ^dUTU-È-A ina KASKAL šu-ut ^dDIŠ ina KI MÚL-MÚL u MÚL.GU₄-AN-NA IGI ana ŠÚ x[...]

x u ina KASKAL šu-ut ^dBE DIB-DIB-iq

The comet which previously had appeared in the east in the path of Anu in the area of Pleiades and Taurus, to the west [...] and passed along in the path of Ea.

BM 41628: 9' (LBAT 378)

[... š]u-ut ^dBE ina KI PA 1 KÙŠ ina IGI MÚL-BABBAR 3 KÙŠ ana SI NIM x[...] [...] in the path of Ea in the region of Sagittarius, 1 cubit in front of Jupiter, 3 cubits high toward the north [...]

More observations of the comet, including precise dates, may have been recorded on these same tablets, but none are now preserved. As it is part of a monthly summary, the statement in LBAT 380 that the comet 'previously had appeared in the east' suggests an observation in a preceding lunar month.

As both tablets are badly damaged, it is difficult to assess exactly how much information is missing from the original records. In both texts, the details about the comet are contained in a summary statement at the end of a month. However, neither on BM 41462 nor on BM 41628 is the month (or even the year) preserved. Both texts must be dated by astronomical calculation using the lunar and planetary observations that are also recorded.

The obverse of BM 41462, on which the comet is reported, covers much of two consecutive months. Among the observa-

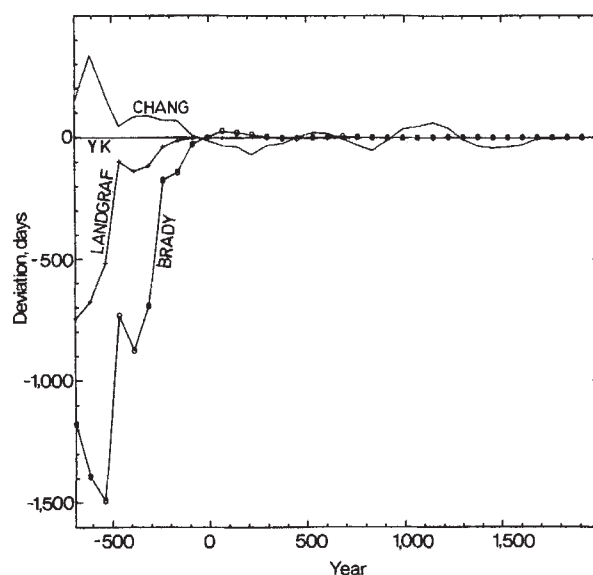


Fig. 2 Plot of the deviations between the calculated dates of perihelion passage for Halley's comet calculated in refs 6, 8, 9 relative to the results of Yeomans and Kiang⁷ for the period since 700 BC. YK, Yeomans and Kiang.

tions that are useful in dating the tablet are the following: (1) During the month in which the comet "passed along in the path of Ea", Jupiter was in Sagittarius; Venus was also in Sagittarius until the middle of the month, afterwards moving into Capricornus; Mercury was in Libra. (2) On the night of the 8th(?) of this same month, in the early evening, Venus was 1 cubit below Jupiter. (The cubit was equivalent to ~2.5°; see below.) (3) On the 14th, towards morning, the Moon was 1.5 cubits in front of β Tau and 4 cubits towards the south of this star. (4) On the 23rd, towards morning, the Moon was 1 cubit in front of γ Vir and 1.5 cubits towards the south. (5) On the 28th, in the early evening, Venus was 2.5 cubits below β Cap. (6) About the 12th of the following month, Venus was 2 fingers (that is, some 0.2°) above γ Cap, whereas towards morning Mars was above α Vir.

On both this tablet and BM 41628, the abbreviations used for the names of stars indicate a date of writing well after 400 BC. No diaries are known later than 40 BC and we have thus limited our investigation to the period between 400 BC and the beginning of the Christian Era. During this time, the only date that satisfies the various astronomical data is the lunar month beginning on 21 October (Julian calendar) in 164 BC. From the tables of Parker and Dubberstein¹³, this is equivalent to month VIII of the year 148 of the Seleucid calendar (that is, SE 148).

The tablet catalogued as BM 41628 is much more extensively damaged than BM 41462. Its obverse again covers portions of two consecutive months, but most of the entries are fragmentary. Nevertheless, several lunar and planetary observations have survived intact. These include: (1) During the month in which the comet was recorded, Saturn was in Scorpius, Mars was in Virgo and, as the cometary text states, Jupiter was in Sagittarius. (2) On the 11th of the same month. Saturn set heliacally in the end of Scorpius. (3) On the 18th, Mercury set heliacally in the east in Scorpius. (4) On the 11th of the next month, at the beginning of the night, the Moon was 1 cubit in front of α Tau and 1 cubit towards the north. (5) About the 12th of this month, Venus was 2 fingers above γ Cap. (See also entry (6) under BM 41462.)

Calculation of these observations leads to precisely the same date, the lunar month beginning on 21 October in 164 BC.

As well as dating the two texts separately, it is important to examine the direct evidence that both tablets share a common date. On each obverse the following duplicate observations are found: (1) Towards the end of the lunar month in which the comet is mentioned, the time interval between moonrise and sunrise at last visibility of the crescent Moon is given as 23,40

uš (that is, 23.7° or ~ 95 min). (2) Attention has already been drawn to the observation on about the 12th of the following month when Venus was 2 fingers above γ Cap.

On the reverse of both tablets there are several identical pairs of observations, both covering two successive months: (1) On the 7th of the second month cited, both texts state that at the beginning of the night the Moon was $2/3$ cubit behind β Gem. (2) On the 9th, both texts assert that at the beginning of the night the Moon was 6.5 cubits below ϵ Leo. (3) On the 15th, in each case the interval between sunrise and moonset is given as 5,30 uš (that is, 5.5° or 22 min). (4) As well as the above, on about the 15th of the month a lunar eclipse is reported. The two texts give few overlapping details but a separate tablet giving fuller information (BM 34037) shows that they represent portions of the same eclipse record.

The evidence is thus conclusive that the two tablets represent copies, albeit with slight variants, of the same astronomical diary. Many similar duplicate and some triplicate texts are known.

(2) 87 BC

BM 41018 rev. 8'-10' (unpublished)

[...] GE₆13(?) 8 ME muš USAN ^dšal-lam-mu-ú

[...] šá ITU.ŠU u₄-mu al-la u₄-mu 1 KUŠ

[...] x bi-rit SI u MAR mi-ši-iš-šú 4 KÜŠ

On the 13th(?), the interval between moonrise and sunset was 8 degrees, measured; first part of the night, a comet [...] which in month IV day beyond day, one cubit [...] between north and west its tail 4 cubits [...]

As indicated by the brackets, the text is fragmentary. The gaps at the beginning of each line may be rather long. The passage quoted is from the 13th(?) (the number is damaged, but certainly it is at least the 12th) day of a month. Although, as before, neither the month nor the year are preserved, both may be derived uniquely from other astronomical data recorded on the same tablet. This information includes: (1) 3rd day of the same month, the Moon was 2 cubits east of Venus; (2) on about the 4th day, Mercury set in the east in Leo; (3) some time between the 4th and 12th Mars was 6 fingers above δ Cnc; (4) 13th day, a lunar eclipse which "passed by", that is, a predicted eclipse which was invisible in the longitude of Babylon; (5) some time between the 13th and 19th, Venus was 1 cubit below α Lib.

The reverse of the text mentions the capital city of Seleucia, founded in 274 BC, which sets a useful time limit. The only astronomically acceptable date is the lunar month beginning on 12 August in 87 BC, that is, month V of the year SE 225. This date is confirmed by the retrospective entry relating to month IV in the account of the comet. Calculation of the time interval between moonrise and sunset on the evening of the cometary observation (that is, 8° or 32 min) determines the date as 24 August, corresponding to the 13th of the lunar month¹³. The fact that the entry for a specific day summarizes observations going back to the previous month strongly suggests that on this day the comet had either reappeared after a period of invisibility or had been seen for the last time.

Because of the gaps in the text, one cannot be definite about certain points of syntax. Thus 'one cubit' may refer to the average daily motion of the comet or it may relate to a verb in the missing part of the next line. However, given the standard presentation of Babylonian observations in which the time reference precedes statements of position or movement, it seems probable that the present passage describes the average daily movement of the comet. The term mishu, which occasionally refers to some luminous phenomenon associated with stars, here seems to indicate the tail of the comet; passages in other texts show that it is usual to specify its direction, as indicated here.

The 'cubit', mentioned in two of the three texts, was used in Babylonian astronomy as an angular unit. It is usually estimated to be $\sim 2^\circ$ (ref. 14). From an analysis of some 20 Babylonian observations of conjunctions of the Moon with stars contained in a diary dating from SE 60 (= 252 BC)¹⁴, we have arrived at the result that 1 cubit = $2.5 \pm 0.5^\circ$ (s.d. per observation). The analysis also indicates that the term ina IGI ('in front of') used

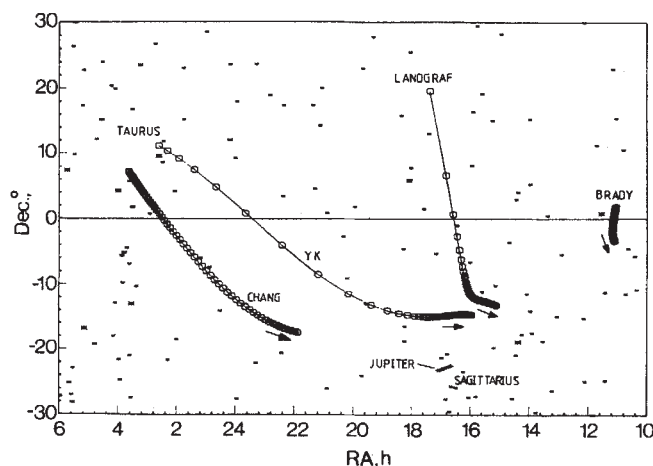


Fig. 3 The computed motion of Halley's comet in 164 BC between 22 Sep and 19 Nov for the dates of perihelion passage deduced by Brady⁸ (164 BC 22 June), Landgraf⁹ (164 BC 30 Oct), YK (Yeomans and Kiang)⁷ (164 BC 12 Nov) and Chang⁶ (163 BC 20 Jan). Positions are at daily intervals. Background stars down to mag +3.5 are shown.

in the second of the 164-BC texts was a standard expression meaning 'to the west of', implying an earlier meridian transit.

The Babylonians divided the sky into three regions of declination named after gods: Enlil, Anu and Ea¹⁵. Of these, Anu was the equatorial region, extending between about $+17^\circ$ and -17° . The entire sky further north was known as Enlil, whereas that to the south was Ea. The limiting declination was based on the well known text ^{mul}APIN (possibly composed ~ 1000 BC), which states that the Sun spent exactly one quarter of a year in each of these regions before moving on to the adjacent one. However, it would perhaps be unwise to assume too precise a definition of Anu and Ea in analysing the motion of the comet.

Analysis of 164 BC observation

There is a brief Chinese record of a comet on a date equivalent to 6 February 162 BC (ref. 5), but this is much too late for Halley's comet. The dates of perihelion for P/Halley as computed in refs 6-9 all lie between June 164 BC and January 163 BC. The salient points of the two Babylonian accounts are as follows: (1) First appearance in the east in the Taurus region, some time before month VIII (21 October to 19 November). (2) Further appearance in the west in Sagittarius during month VIII. (3) As the difference in RA between Taurus and Sagittarius is as much as 14 h in the direct sense, it seems probable that the motion of the comet was retrograde. (4) The position relative to Jupiter (then in Sagittarius, according to calculation) was recorded at some time between 21 October and 19 November (the Babylonian month VIII). The fact that a difference in longitude (some 2.5°), as well as latitude is mentioned, indicates that this is not a record of closest approach. The reason for recording this rather careful measurement is obscure.

Figure 3 is a chart of the sky in the range of RA from 10 to 6 h and dec. from $+30^\circ$ to -30° at the epoch 164 BC. The region of Taurus (where the comet seems to have been first observed) and Sagittarius (where it was later observed) are marked. The range of positions computed from the JPL Ephemeris DE102 (ref. 16) for Jupiter during month VIII is indicated by crosses. The four roughly vertical tracks (composed of small squares) denote the computed motion of Halley's comet during months VII and VIII (22 September-19 November) on each of the dates of perihelion passage deduced by Brady (22 June), Landgraf (30 October), Yeomans and Kiang (12 November), all in 164 BC, and Chang⁶ (20 January 163 BC). In each case the general direction of travel is from north to south, the individual squares representing positions of the comet at daily intervals. Only on the solution of Yeomans and Kiang⁷ does the computed path approximate to the observational record. It is evident that merely

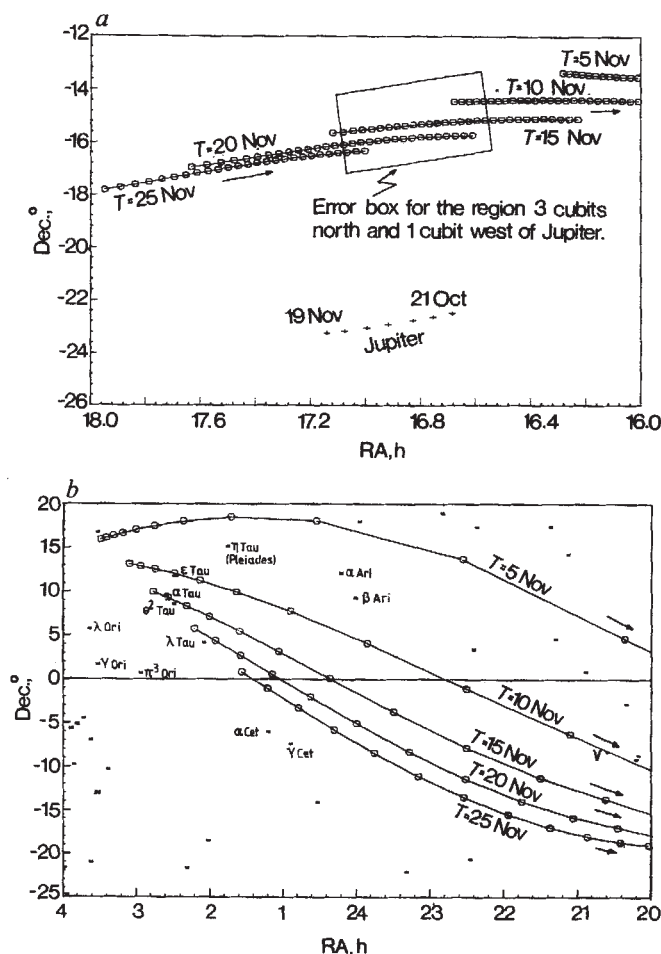


Fig. 4 *a*, Computed paths of Halley's comet in 164 BC between 21 Oct and 19 Nov (month VIII) for dates of perihelion (T) between 5 Nov and 25 Nov. The motion of Jupiter in this interval is shown and also the region of sky 3 cubits ($7.5 \pm 1.5^\circ$) north and 1 cubit ($2.5 \pm 0.5^\circ$) west of the planet as observed by the Babylonians. *b*, Computed paths of Halley's comet in 164 BC from first visibility for T between 5 Nov and 25 Nov. Stars in the Taurus region, where the comet appeared, are labelled.

varying the date of perihelion passage of Halley's comet can achieve a highly satisfactory representation of the observed motion, including the close approach to Jupiter whilst in Sagittarius. In our opinion, this is sufficient grounds for a firm identification of the recorded comet with P/Halley.

Because Halley's comet passed unusually close to the Earth (some 0.1 AU for the date of perihelion deduced by Yeomans and Kiang), small changes in T appreciably alter the relative motion of the two bodies. This circumstance is helpful in fixing narrow limits to the range of dates over which the perihelion can actually lie. We shall first consider the close approach to Jupiter during month VIII (21 October–19 November).

Figure 4a covers the region of sky from RA 16 to 18 h and dec. -12° to -26° at 164 BC. The computed motion of Jupiter (eastwards) during month VIII and the region 3 cubits ($7.5 \pm 1.5^\circ$) north and 1 cubit ($2.5 \pm 0.5^\circ$) west of the planet are shown. Following Babylonian practice, the relative positions of comet and planet are assumed to be measured on the ecliptic system, but there would be little difference if an equatorial frame had been used.

Computed tracks of Halley's comet moving from east to west during month VIII are shown in Fig. 4a for the following dates of perihelion: Nov. 5, 10, 15, 20 and 25 (each at 0 h ET). To agree with observation, some part of the computed path of the comet should intersect the error box. The indicated range of T is thus between 9 and 26 November. If T is altered by one day, the resultant east–west displacement of P/Halley is some 1.2°

so that any observational error in the relative position of the comet and Jupiter can probably be neglected. It seems probable that the original tablet would record the precise date of conjunction with Jupiter and it is regrettable that because of the loss of this information only a range of T covering 17 days can be deduced. Nevertheless, this is much smaller than the scatter among the various dates derived from numerical integration.

As mentioned earlier, Jupiter was only a little to the east of the Sun during month VIII and the visibility of the comet needs to be considered. Based on our investigation of Chinese observations of the heliacal setting of P/Halley (see below under the analysis of the 87 BC record) we conclude that the comet should have been visible to the unaided eye whilst near the marked region. Hence it is not possible to restrict further the range of T on the grounds of limited visibility.

The observation made “in the east . . . in the area of Pleiades and Taurus” is less accurate but gives an independent result. The precise date is no longer extant but it must be earlier than month VIII. Far-eastern records indicate that Halley's comet rarely remains visible to the unaided eye for >50 days so that first sighting is unlikely to have been earlier than month VI. During months VI and VII, Taurus would be high in the eastern sky at about midnight and the comet would thus be well placed for viewing in a dark sky. Figure 4b is a chart of the sky in the range RA 20 to 4 h and dec. -25° to $+20^\circ$. For each of the following dates of perihelion: 5, 10, 15, 20 and 25 November, the computed motion of Halley's comet is shown. The individual tracks begin with the location of the comet at what we infer to be its earliest probable date of discovery. We have investigated the discovery magnitude of P/Halley throughout much of its history using some 20 dates of first sighting reported in various far-eastern records. From the empirical formula of Morris and Green¹⁷, based on the observations in 1910, we find that the comet is seldom detected with the unaided eye even in a dark sky until it becomes fairly bright, between about $+3.5$ and $+4$ mag. (There is only a single instance of discovery when the calculated magnitude was fainter than $+4$, that is, $+4.3$ mag in 12 BC.) This is, of course, well above the threshold brightness for the eye (around $+6$ mag) but these early observers, unlike their modern counterparts, had no advance warning. We have thus assumed a discovery magnitude of $+4$ at Babylon in 164 BC.

Before attempting to refine the range of T already deduced, the identification of MÚL-MÚL (rendered above as Pleiades) and MÚL.GU₄-AN-NA (Taurus) must be considered. The modern equivalents of the various Babylonian star groups have been investigated in detail by Kugler¹⁸, Schaumberger¹⁵, Gössmann¹⁹ and, more recently, Reiner²⁰. From these works it is apparent that MÚL-MÚL (‘The Stars’) was identical to the Pleiades group whereas MÚL.GU₄-AN-NA (‘The Bull of Anu’) was centred on α Tau and the Hyades.

From the close approach to Jupiter, a date of perihelion passage for Halley's comet between 9 and 26 November has already been derived. Referring to Fig. 4b, it is not possible to use the Pleiades/Taurus observation to refine the earlier of these two limits, as for any T between about 7 and 16 November P/Halley would pass directly between η Tau and α Tau/Hyades. However, for dates of perihelion after about 24 November, the comet would probably not be noticed until it had moved into Cetus (MÚL-ĪKU). Hence we can now place firm limits on the date of perihelion for Halley's comet in 164 BC between 9 and 26 November, the latter limit possibly being as early as 24 November.

Analysis of 87 BC observation

Apart from the Babylonian text, there is a brief Chinese report of a comet appearing during this year which may relate to P/Halley^{2,3}. However, only the month and general area of sky in which the comet appeared are given and we shall not consider this observation further. It is unfortunate that the cuneiform record is also brief. Despite this, several important deductions can be made. These may be summarized as follows: (1) As suggested above, there are good reasons for believing that the

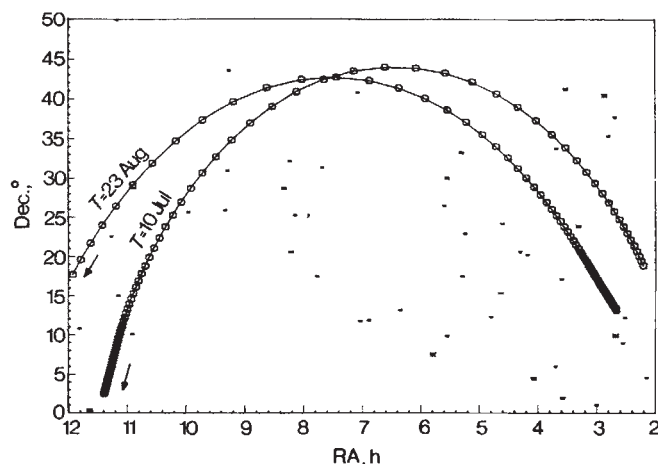


Fig. 5 The computed motion of Halley's comet in 87 BC for both the lower and upper limits of the theoretical T , that is 10 Jul (ref. 8) and 23 Aug (ref. 9) over the time range 13 Jun to 9 Sep (months III–V).

observation reported on 24 August represented either reappearance or last visibility. Such a circumstance is unlikely to result from unfavourable weather, for during the summer months the skies are almost cloudless in this part of the world. (2) The comet was seen 'day beyond day' (u_4 -mu al-la u_4 -mu) during the 4th lunar month (14 July–11 August), which seems to imply a lengthy period of visibility during this month. (3) We add that the probability of our text referring to P/Halley, rather than some other comet, is high. The various computed dates of perihelion passage lie in the range 10 July–23 August. Calculation shows that on any of these solutions, Halley's comet would be well placed for northern observers during the summer of 87 BC (Fig. 5) and fairly bright, approaching 0 mag according to the formula of Morris and Green¹⁷.

Figure 5 shows the computed motion of Halley's comet in a general easterly direction during months III, IV and V (13 June–9 September) in 87 BC on the dates of perihelion computed by Brady⁸ (10 July) and Landgraf⁹ (23 August). These represent the extreme calculated dates; Yeomans and Kiang⁷ deduced 6 August, whereas Chang⁶ gave 15 August. For any of these values of T , the comet never approached very close to the Earth (minimum distance ~ 0.45 AU) so that the motion was unusually regular. The calculated daily motion, indicated by small squares on the diagram, was always $>1^\circ$ and $<6^\circ$ for at least a month. As both of these values are within about a factor of two of 1 cubit, this seems to explain the reference to this quantity in the text, that is, 'day beyond day one cubit'.

As the displacement in the position of P/Halley in response to a change in T was fairly linear, it is possible to summarize the main characteristics of the apparent motion almost independently of the precise date of perihelion. On any of the four solutions cited, the comet would first reach the estimated limiting discovery brightness ($+4$ mag) while some 45° west of the Sun. It would then be fairly high in the eastern sky before dawn. Moving quickly eastwards, the comet would pass conjunction with the Sun approximately 10–15 days later, now becoming an evening object. Gradually diminishing in speed, it would reach a maximum elongation of only $\sim 35^\circ$ east of the Sun after a further 15 days or so. Some time later the comet, approaching a second conjunction with the Sun and growing progressively fainter, would probably set heliacally, being lost to view in the evening twilight.

From the above outline, it is apparent that the Babylonian record indicates observations both before and after the first conjunction with the Sun. The tail could only have pointed north-west before this conjunction. However, on 24 August, the date of the preserved diary entry, the comet was said to be visible in the "first part of the night" and thus to the east of the

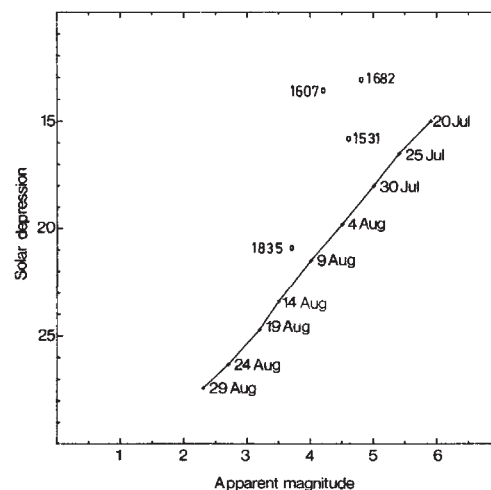


Fig. 6 Plot of the solar depression as a function of the calculated apparent magnitude of P/Halley on the evening of 24 Aug in 87 BC for dates of perihelion between 20 Jul and 29 Aug with, for comparison, the circumstances at the last recorded visibility (in Korea) in AD 1531, 1607, 1682, 1835.

Sun. (The Babylonian astronomers divided the interval between sunset and sunrise into three roughly equal parts.) On the basis of brightness considerations, it can be deduced that by 24 August Halley's comet was probably well past conjunction with the Sun. For the comet to have been just emerging after conjunction would require an estimated discovery date ($+4$ mag) no earlier than about 13 August. As month IV ended on 11 August and the computed magnitude was changing by about 0.2 daily, there would seem to be no possibility of observation 'day beyond day' during this month under these circumstances. Thus, we infer that the most probable interpretation of the observation on 24 August is final visibility.

Before analysing the 24 August observation, we note that it is possible to fix useful limits on the date of perihelion in 87 BC from the additional information supplied in the Babylonian record. For T before about 20 July, the tail of Halley's comet could only have pointed north-west earlier than month IV, but such an early date does not seem to be implied in the text. If T was after about 30 August, the period of visibility in month IV would probably be too short to be described as 'day beyond day'. These limits may be narrowed further on the assumption of last sighting on 24 August.

Figure 6 illustrates the circumstances relating to the visibility of P/Halley on the evening of 24 August in 87 BC for dates of perihelion between 20 July and 29 August. The abscissa gives the apparent magnitude of the comet as deduced from the empirical formula of Morris and Green¹⁷. The ordinate is the depression of the Sun below the western horizon at the moment when the nucleus of the comet set (refraction being ignored). For the earlier dates of perihelion, the comet would set in strong twilight. The best time for observation would then be roughly midway between sunset and the setting of the comet itself, when the effects of the twilight glow and atmospheric absorption were optimized (although the tail would then be pointing in a roughly vertical direction, it would be very short on account of the comet's considerable distance from the Earth, some 1.5 AU for T before about 1 August).

The precise date of heliacal setting for a celestial body depends on various factors (largely of atmospheric origin) and is therefore best treated empirically. To provide some parallel data, we have analysed several dates of the last sighting of P/Halley as reported in far Eastern history. Such information is fairly regularly available over the past two millennia but before $\sim$ AD 1400 only summary records are now extant. Thus, there is a distinct possibility of copying errors, but for later centuries detailed chronicles are available from both China and Korea. The Korean works are particularly useful for our purpose because they contain day-to-day reports of astronomical phenomena, usually

stating when cloud prevented observation. Calculations based on the accurately known dates of perihelion at this relatively late period enable several instances of heliacal setting to be identified. The dates of these are 1835 November 17, 1682 October 7, 1607 November 7 and 1531 September 15. In each case the computed magnitude and solar depression are plotted in Fig. 6 (represented by circles).

Reference to Fig. 6 shows that there is some scatter among the Korean data, the 1835 solution lying somewhat below the others. The most probable explanation involves variations in the intrinsic brightness of Halley's comet from one apparition to the next, although the oriental records of first visibility show no significant long-term trends²¹. It is not possible to make any accurate deductions regarding perihelion in 87 BC, but the following remarks seem relevant: (1) Unless Halley's comet was unusually bright in 87 BC, the date of perihelion is unlikely to have been before about 25 July. For earlier dates the computed magnitude on 24 August was close to the normal limit for the unaided eye while atmospheric absorption (some 1.5 mag at optimum visibility) and the twilight glow would probably reduce the brightness well below the visual threshold. (2) For dates of perihelion after about 15 August both the calculated apparent magnitude and solar depression were very favourable and there should have been no difficulty in observing the comet on 24 August.

In summary, we infer a likely date of perihelion in 87 BC between about 25 July and 15 August. If we have misinterpreted the nature of the observation on 24 August, the latter limit would, of course, be invalid and perihelion might then lie anywhere between 25 July (to render the comet visible at all on the recorded date) and the end of August. However, the mere fact that in a day-to-day diary covering (originally) several

months the Babylonian astronomers felt it sufficiently important to provide a detailed retrospective entry on a specific day (24 August) seems consistent with an abrupt change in the visibility of the comet at that time; this we have presumed to be heliacal setting.

Conclusions

We believe that the evidence that Halley's comet was observed in Babylon at its apparitions in both 164 and 87 BC is convincing. The records, although badly damaged, enable fairly narrow limits to be placed on the dates of perihelion in both years, between about 9 and 24 November in 164 BC and between about 25 July and 15 August in 87 BC. Of the four numerical integrations investigated here, only that of Yeomans and Kiang⁷ provides acceptable dates of perihelion in both years. This presumably results from the incorporation in their solution of several rather accurate ancient and medieval observations, rather than using a purely theoretical analysis. In particular, their identification of a brief Chinese record in 240 BC as the most ancient reliable sighting of the comet finds support here. The Babylonian observations may prove useful in the integration of the motion of P/Halley further backwards in time, possibly leading to the recognition of still earlier records of this most famous of all comets.

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1. Halley, E. *Phil. Trans. R. Soc.* **24**, 1882 (1705).
2. Kiang, T. *Mem. R. astr. Soc.* **76**, 27-66 (1972).
3. Stephenson, F. R. & Yau, K. K. C. *J. Brit. Interplanet. Soc.* (in the press).
4. Sachs, A. J. *Late Babylonian Astronomical and Related Texts* (Brown University Press, Providence, 1955).
5. Ho Peng Yoke *Vistas Astr.* **5**, 127-225 (1962).
6. Chang, Y. C. *Chin. astr.* **3**, 120-131 (1979).
7. Yeomans, D. K. & Kiang, T. *Mon. Not. R. astr. Soc.* **197**, 633-646 (1981).
8. Brady, J. L. *J. Brit. astr. Assoc.* **92**, 209-215 (1982).
9. Landgraf, W. *Astr. Astrophys.* (in the press).
10. Sachs, A. J. *J. Cuneiform Studies* **2**, 271-290 (1948).
11. Sachs, A. J. *Phil. Trans. R. Soc.* **A276**, 43-50 (1974).

12. *The Chicago Assyrian Dictionary* Vol. 16, 74-75 (Chicago, 1962).
13. Parker, R. A. & Dubberstein, W. H. *Babylonian Chronology: 626 BC to AD 75* (Brown University Press, Providence, 1956).
14. Aaboe, A. *Centaurus* **24**, 14-35 (1980).
15. Schaumberger, J. *Sternkunde und Sterndienst in Babel—Ergänzungsheft 3* (Aschendorffsche, Münster, 1935).
16. Newhall, X. X., Williams, J. G. & Standish, E. M. *Astr. Astrophys.* **125**, 150-167 (1983).
17. Morris, C. S. & Green, D. W. E. *Astr. J.* **87**, 918-923 (1982).
18. Kugler, F. X. *Sternkunde und Sterndienst in Babel* (Aschendorffsche, Münster, 1907-1924).
19. Gössmann, F. in *Sumerisches Lexikon* (IV, 2) (ed. Deimel, A.) (Pontifical Biblical Institute, Rome, 1950).
20. Reiner, E. *Babylonian Planetary Omens*, in G. Buccellati (ed.), *Bibliotheca Mesopotamica* (II, 2), Malibu (1981).
21. Stephenson, F. R. & Yau, K. K. C. *New Scient.* **103**, 30-32 (1984).

Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase

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The products of six unidentified reading frames of human mitochondrial DNA are precipitated from a mitochondrial lysate by antibodies against highly purified native beef heart NADH-ubiquinone oxidoreductase (complex I). These products are enriched greatly in a human submitochondrial fraction enriched in NADH-Q₁ and NADH-K₃Fe(CN)₆ oxidoreductase activities. We conclude that the six reading frames encode components of the respiratory-chain NADH dehydrogenase.

ONE of the most remarkable findings from the sequence analysis of mitochondrial DNAs from several mammalian sources¹⁻⁴, *Xenopus laevis* (B. Roe, personal communication) and

Drosophila species⁵⁻⁷ has been the discovery that more than half of the informational content of animal mtDNA resides in eight unidentified reading frames (URFs). These genes, with one

exception, have no obvious amino-acid sequence homology to any yeast mtDNA reading frames. The demonstration that URFs with partial homology to several of the mammalian mtDNA URFs occur in mtDNA from other lower eukaryotic organisms, as first observed in *Aspergillus nidulans*^{8,9} and then in *Neurospora crassa*⁹ and protozoa^{10,11}, strongly supported the idea that these URFs code for functional proteins. Direct evidence that the URFs are functional genes has been provided by the identification of translation products corresponding to most of the human URFs, achieved by the use of antibodies directed against peptides predicted from the DNA sequence. This approach has led to the identification of the polypeptides encoded in URFA6L (ref. 12), URF1 (ref. 13), URF3 (ref. 13), URF2, URF4 and URF4L (P.M., A.C., M. Riley, B. Cottrell, R.F.D. and G.A., in preparation).

Little has been discovered about the physiological role of mtDNA URF products from animal and other species. The product of the smallest URF, URFA6L, has a low but significant amino-acid sequence homology and other structural similarities^{14,15} to the product of a newly identified yeast mtDNA gene (*aap1*)¹⁴, which seems to be a component (subunit 8) essential for the assembly of a functional H^+ -ATPase complex; these observations have suggested that the URFA6L product is associated with the mitochondrial H^+ -ATPase^{14,15}. The other URF products are hydrophobic proteins¹⁶ and, therefore, are presumably associated with the inner mitochondrial membrane. Among the enzyme complexes of this membrane, the NADH-ubiquinone oxidoreductase (complex I, ref. 17) and the succinate-ubiquinone oxidoreductase (complex II, ref. 18) seemed to be good candidates for containing some of the URF products, despite the previous failure to detect mitochondrially synthesized polypeptides in NADH-ubiquinone oxidoreductase of rat source¹⁹. The NADH-ubiquinone oxidoreductase (hereafter referred to as respiratory-chain NADH dehydrogenase or complex I) was of particular interest because it consists of a shell of ≥ 15 hydrophobic polypeptides surrounding a core containing the low-relative molecular mass (M_r) NADH dehydrogenase or flavoprotein fragment and the iron-protein fragment^{20,21}. Here we describe immunoprecipitation experiments using antibodies raised against purified beef heart complex I or against synthetic peptides predicted from the human URF sequences and enzyme fractionation studies; the results of the three approaches strongly suggest that the products of six of the human URFs, URF1, URF2, URF3, URF4, URF4L and URF5, are components of the respiratory-chain NADH dehydrogenase.

Products of mitochondrial genes

Figure 1 shows the present status of the polypeptide assignment to the reading frames of human mtDNA. Most of these assignments have been made by the use of anti-peptide antibodies; cytochrome *c* oxidase subunits I, II and III (COI, COII and COIII) and cytochrome *b* have been identified by analysis of the site of synthesis and electrophoretic properties of the subunits of purified human cytochrome *c* oxidase complex²² and, respectively, purified human cytochrome *b*-*c*₁ complex (C.-J. Doersen and G.A., in preparation). The mitochondrially synthesized component 1 ($M_r \sim 51,000$; ref. 23) has been identified tentatively as the product of URF5 (expected $M_r = 66,600$; ref. 1), because this is the only gene that could code for such a large polypeptide. The discrepancy between the expected and the estimated size can be explained by the well-known bias in size estimates based on SDS-polyacrylamide gel electrophoresis of mtDNA-encoded hydrophobic proteins^{12,13}. Thus, it seems that the main translation products of the human mitochondrial genes have been identified.

Reaction with anti-peptide antibodies

To determine whether the URF products are part of a protein complex, several individual URF-specific anti-peptide antisera were tested on a Triton X-100 HeLa cell mitochondrial lysate; it seemed possible that at least some of the antigenic peptides in such a complex would be accessible to their respective anti-

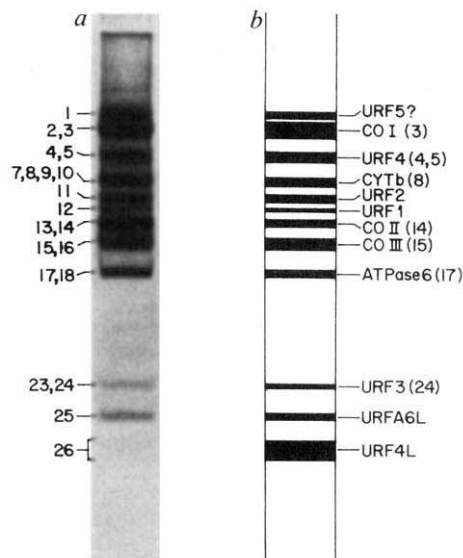


Fig. 1 Polypeptide assignment to the human mitochondrial genes. *a*, Electrophoretic pattern in an SDS/urea-polyacrylamide gel of the HeLa cell mitochondrial translation products labelled *in vivo* with ³⁵S-methionine in the presence of 100 $\mu\text{g ml}^{-1}$ emetine and numbered according to Ching and Attardi²³. *b*, Pattern of the polypeptides assigned to individual reading frames of human mtDNA. Anti-peptide antibodies were used for the polypeptide assignment to URF1, URF3, URFA6L, ATPase 6 (refs 12, 13), URF2, URF4 and URF4L (P.M. *et al.*, in preparation); the polypeptides corresponding to COI, COII and COIII were identified by analysis of the subunits of purified human cytochrome *c* oxidase²², and the product of the cytochrome *b* gene by analysis of the subunits of purified cytochrome *b*-*c*₁ complex (C.-J. Doersen and G.A., in preparation). Numbers in parentheses indicate the polypeptide within each multicomponent band that corresponds to the indicated reading frame.

bodies. Indeed, we found that each antiserum tested had a tendency to precipitate other URF products as well as the polypeptide against which it had been raised. This was seen most clearly with an antiserum against the carboxy-terminal heptapeptide of the URF4L product (anti-URF4L-C). This antiserum precipitated only one component, component 26, from an SDS mitochondrial lysate (Fig. 2*a*), which represents the URF product against which it had been prepared (P.M. *et al.*, in preparation). In striking contrast, the same antiserum precipitated components 1 (URF5), 4 and/or 5 (URF4), 11 (URF2), 12 (URF1) and 24 (URF3) as well as component 26 from Triton X-100 mitochondrial lysate (Fig. 2*b*). The same phenomenon was observed with an antiserum against the carboxy-terminal nonapeptide of the URF4 product (anti-URF4-C) (Fig. 2*a, b*) and with anti-URF2-N and anti-URF3-C antisera (not shown); each of these antisera precipitated its respective polypeptide, as well as a small amount of the other five URF products of the group specified above.

All immunoprecipitates produced from a Triton X-100 lysate by anti-URF4, URF4L, URF2 and URF3 antibodies exhibited a variable, generally relatively small amount of cytochrome *c* oxidase subunits I, II and III, but we did not detect cytochrome *b* or ATPase subunits 6 and 8 (URFA6L product). It seems probable that the presence of cytochrome *c* oxidase subunits in the specific precipitates reflects an incomplete dissociation or a secondary aggregation of these subunits in the experimental conditions used here. Even so, the tendency of the products of URFs 1, 2, 3, 4, 4L and 5 to be co-precipitated by the individual anti-peptide antibodies suggested that these components are associated with each other in some kind of complex.

Reaction with anti-complex I antibodies

To test the possibility that the putative complex containing URF1, URF2, URF3, URF4, URF4L and URF5 products is the respiratory-chain NADH dehydrogenase (complex I), we used

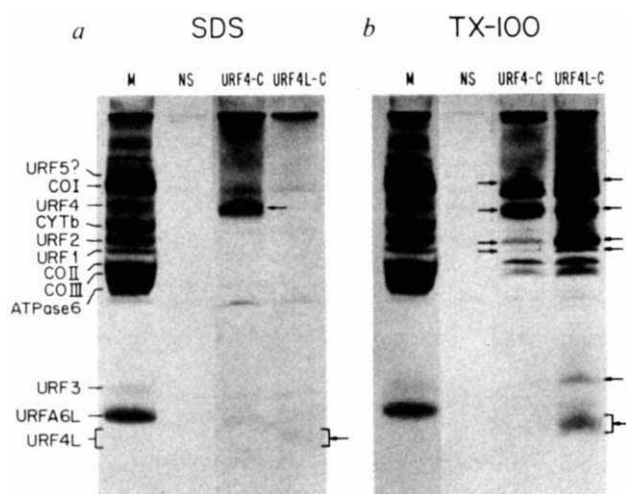


Fig. 2 Co-precipitation of several URF products by individual URF-specific antipeptide antisera. Shown are fluorograms, after electrophoresis through SDS/urea-polyacrylamide gels, of HeLa cell mitochondrial translation products immunoprecipitated by antibodies against peptides corresponding to specific regions of URF4 and URF4L. γ -Globulins from antisera against the carboxy-terminal nonapeptide of the URF4 product (URF4-C) or the carboxy-terminal heptapeptide of the URF4L product (URF4L-C) or from normal serum (NS) were incubated with an SDS lysate (a) or a 0.5% Triton X-100 (TX-100) lysate (b) of 35 S-methionine-labelled mitochondria. M, Pattern of mitochondrial translation products.

Methods. Antisera were prepared as described elsewhere (ref. 12 and P.M. *et al.*, in preparation). A γ -globulin fraction, free of anti-bovine serum albumin antibodies¹², was used in these experiments. For the selective labelling of the mitochondrial translation products, HeLa cells grown for 22 h in the presence of $40 \mu\text{g ml}^{-1}$ chloramphenicol²⁷ were labelled with 35 S-methionine ($1,040 \text{ Ci mmol}^{-1}$; $19 \mu\text{Ci ml}^{-1}$) for 2 h in the presence of $100 \mu\text{g ml}^{-1}$ cycloheximide in methionine-free medium supplemented with 5% dialysed calf serum, then pelleted, washed once in complete medium, resuspended in the same medium at 10^5 cells per ml, and incubated for an additional 18 h. The mitochondrial fraction was prepared as described previously¹². a, An SDS lysate of the mitochondrial fraction was prepared and aliquots (2×10^5 c.p.m., $220 \mu\text{g}$) were immunoprecipitated at room temperature with $250 \mu\text{g}$ of γ -globulins from each of the antisera or from NS by a modification of the previously described procedure¹², to be described in detail elsewhere (P.M. *et al.*, in preparation). The immunoprecipitated products were run on an SDS/urea-polyacrylamide gel¹². b, For immunoprecipitation of intact complexes of the inner mitochondrial membrane, all operations were carried out at 4°C . Samples of mitochondrial suspension (2×10^5 c.p.m., $220 \mu\text{g}$) were resuspended in $50 \mu\text{l}$ 0.005 M Tris-HCl, pH 6.7 (25°C), 0.002 M methionine, 0.001 M phenylmethylsulphonyl fluoride, 0.5% (w/v) Triton X-100, at a concentration of $5 \text{ mg protein ml}^{-1}$ and incubated for 20 min. After diluting the samples fourfold with the same solution containing 1 mg ml^{-1} ovalbumin (incubation buffer), methanol was added to 2% and the mixtures incubated for a further 10 min. After addition to each sample of $125 \mu\text{l}$ formaldehyde-fixed *Staphylococcus aureus*, Cowan strain I suspension (Zysorbin, Zymed), prewashed with and suspended in incubation buffer, the mixtures were incubated for 30 min and then centrifuged in an Eppendorf microfuge for 2 min. To each supernatant, $70 \mu\text{l}$ incubation buffer and $250 \mu\text{g}$ γ -globulin fraction in $70 \mu\text{l}$ phosphate-buffered saline were added and the mixtures incubated for 1 h. After addition of a 10-fold protein excess of a 0.5% Triton X-100 mitochondrial lysate (12.5 mg ml^{-1}) from unlabelled cells and SDS to 0.1% , the samples were mixed with $125 \mu\text{l}$ Zysorbin suspension, incubated for 30 min and centrifuged in the microfuge for 2 min. The pellets were washed by centrifugation once with incubation buffer containing 0.1% SDS, once with incubation buffer and once with 0.010 M Tris-HCl, pH 6.7 and then extracted with $60 \mu\text{l}$ 8 M urea, 4% SDS, 0.01 M Tris-HCl, pH 7.4, 0.1% β -mercaptoethanol for 30 min at 37°C and 10 min at 50°C . The extracts were run on an SDS/urea-polyacrylamide gel.

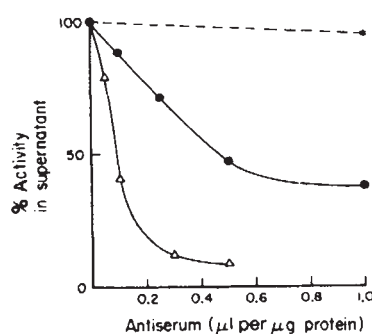


Fig. 3 Immunoprecipitation of complex I from solubilized human kidney submitochondrial particles by antiserum 1 against native beef heart complex I. ●, Antiserum tested on human kidney submitochondrial particles; Δ, antiserum tested on beef heart submitochondrial particles; *, normal serum tested on human material.

Methods. Submitochondrial particles were prepared from human kidney or beef heart mitochondria and solubilized with 0.5% Na-DCC in the presence of 0.5 M KCl, as described previously²⁵. After centrifugation in the $10 \times 10 \text{ ml}$ rotor of an MSE 65 centrifuge at $25,000 \text{ r.p.m.}$ for 40 min, the supernatant was incubated at 4°C overnight in the presence of 1% Triton X-100. After another centrifugation as above, portions of the supernatant ($50 \mu\text{g}$ protein in $50 \mu\text{l}$) were incubated for 1 h at 4°C with the indicated volumes of antiserum 1 against beef heart complex I or normal serum (with addition of Triton X-100 to a final concentration of 1%). Samples were assayed for NADH- $\text{K}_3\text{Fe}(\text{CN})_6$ oxidoreductase activity²⁵ after centrifugation in the conditions described above.

rabbit antisera raised against highly purified native bovine complex I (ref. 24). One such antiserum (antiserum 1) could precipitate $\sim 60\%$ of the NADH- $\text{K}_3\text{Fe}(\text{CN})_6$ oxidoreductase activity from deoxycholate- and Triton X-100-solubilized human kidney-submitochondrial particles (Fig. 3). The non-precipitated enzyme activity results from an unrelated enzyme with different kinetic properties^{25,26} which is more abundant in the kidney ($40\text{--}50\%$) than in the heart ($10\text{--}20\%$) (C.I.R., unpublished observations). Electrophoretic analysis of the immunoprecipitated human kidney complex I on a $12\text{--}16\%$ polyacrylamide gel, in parallel with the beef heart complex I, revealed a profile very similar to that of the beef enzyme, with several recognizable main components in the high- M_r range ($>25,000$). The human complex I profile diverged from the beef enzyme profile in the low- M_r region, in agreement with previous observations on rat and rabbit complex I (M.W.J.C. and C.I.R., unpublished observations). In immunoblot assays with SDS-denatured human kidney submitochondrial particles, antiserum 1 showed a wide reactivity with human proteins, revealing at least 12 components in the M_r range $10,000\text{--}50,000$.

The antiserum described above was tested on an SDS mitochondrial lysate from HeLa cells grown for 22 h in the presence of $40 \mu\text{g ml}^{-1}$ chloramphenicol²⁷, labelled with 35 S-methionine in the presence of $100 \mu\text{g ml}^{-1}$ cycloheximide and chased in unlabelled medium for 18 h. Only weak bands corresponding to the product of URF4 and to the putative product of URF5 were detected in the autoradiogram of the immunoprecipitate fractionated on a gel, despite the large amount of radioactivity used in this experiment (Fig. 4a, lane 3); these bands, however, were specific, because they were absent or much weaker in the control precipitate obtained with normal serum γ -globulins. A more dramatic result was obtained when the same antiserum was tested on a Triton X-100 mitochondrial lysate from the same cells. Six URF products were precipitated in these conditions, that is, the products of URF1, URF2, URF3, URF4, URF4L and URF5, with a minimal degree of contamination by COI, COII and COIII (Fig. 4b, lane 6). In the same conditions, antibodies against the COOH-terminal undecapeptide of COII precipitated COI, COII and COIII (Fig. 4b, lane 7); these are part of the intact cytochrome c oxidase complex

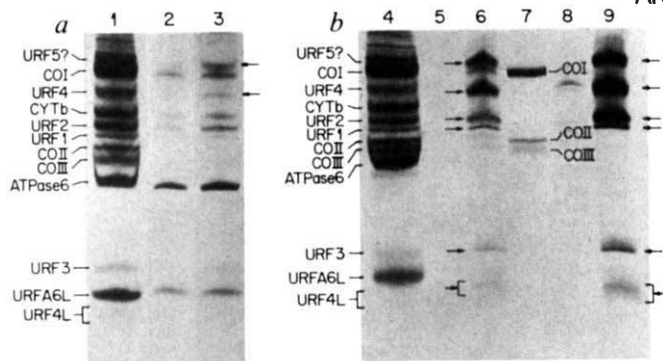


Fig. 4 Antibodies against native bovine complex I immunoprecipitate several URF products. *a*, Immunoprecipitates from an SDS lysate of HeLa cell mitochondria incubated with normal serum γ -globulins (lane 2) or with antiserum 1 against beef heart complex I (lane 3). *b*, Immunoprecipitates from a 0.5% Triton X-100 lysate of HeLa cell mitochondria incubated with normal serum γ -globulins (lane 5), or with antiserum 1 against beef heart complex I (lane 6), or with γ -globulins from an antiserum against the carboxy-terminal undecapeptide of COII (COII-C, ref. 12; lane 7). Lanes 8 and 9 show immunoprecipitates from a 0.2% DOC mitochondrial lysate incubated with normal serum γ -globulins or with antiserum 1 against beef heart complex I, respectively. Lanes 1 and 4, pattern of mitochondrial translation products.

Methods. *a*, Samples of a mitochondrial suspension (10^6 c.p.m., 550 μ g protein) from HeLa cells labelled as described in Fig. 2 legend and chased for 2 h were solubilized with SDS and exposed to 50 μ l of antiserum 1 against native beef heart complex I or to 500 μ g normal serum γ -globulins, as in the experiment described in Fig. 2a legend. *b*, Samples of a mitochondrial suspension (2×10^5 c.p.m., 215 μ g protein) from cells labelled and chased as described in Fig. 2 legend were solubilized with 0.5% Triton X-100 and immunoprecipitated with 25 μ l antiserum 1 (lane 6), or with 250 μ g γ -globulins from anti-COII-C antiserum (lane 7), as described in Fig. 2b legend. An identical sample (lane 9) was solubilized in 25 μ l solution containing 0.2% DOC instead of 0.5% Triton X-100; after 20 min at 4°C, KCl was added to 0.15 M; after a further 10 min, 65 μ l of Zysorbin suspension in incubation buffer (adjusted to 0.15 M KCl) were added and the mixture incubated for 30 min at 4°C, then spun in the microfuge. The supernatant, after addition of 80 μ l of incubation buffer and 25 μ l antiserum 1, was incubated for 1 h and then mixed with 125 μ l of a 0.5% Triton X-100 mitochondrial lysate (12 mg protein ml⁻¹) from unlabelled cells, then SDS to 0.1% and finally 65 μ l Zysorbin. At this stage, the sample contained ~0.015% DOC and ~0.5% Triton X-100. Further treatment of the sample was identical to that of the Triton X-100-solubilized sample. As controls, equivalent portions of the Triton X-100 (lane 5) or DOC-solubilized mitochondrial suspension (lane 8) were incubated with 250 μ g γ -globulins from normal rabbit serum. Exposure of the autoradiograms was 10 (*a*) or 12 (*b*) days.

(P.M. *et al.*, in preparation). No URF products were observed in the precipitate obtained with normal serum γ -globulins (an independent control experiment showed that complete normal serum gave the same results as normal serum γ -globulins). From the densitometer tracing of the autoradiogram and the methionine content of the various URF products, the labelled products of URF5, URF4, URF2, URF1, URF3 and URF4L in the immunoprecipitate had estimated relative molar concentrations of ~5, 5, 2, 1, 1 and 0.6. In a control experiment, the labelling of all the immunoprecipitated components was sensitive to chloramphenicol.

A more effective precipitation of all six URF products by anti-complex I antibodies was obtained when 0.2% potassium deoxycholate (DOC) in the presence of KCl was used to solubilize the mitochondrial membranes (Fig. 4b, lane 9). In these conditions, the relative molar concentrations of the labelled URF products in the immunoprecipitate were similar to those observed in the Triton X-100-solubilized sample (~3, 3, 2, 0.7, 1 and 0.5 for the six polypeptides, respectively). In this last

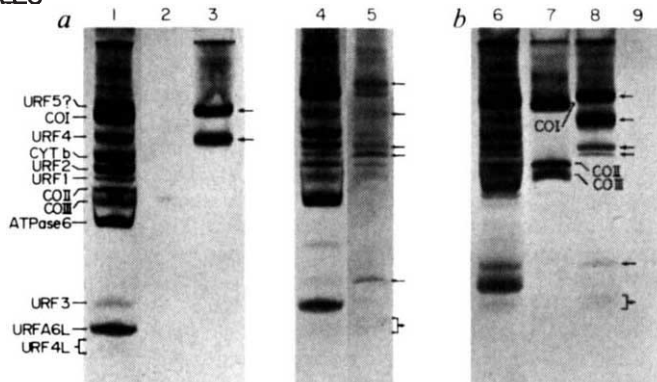


Fig. 5 Effect of detergent treatment (*a*) and labelling conditions (*b*) on the precipitation pattern of URF products by anti-complex I antibodies. *a*, Immunoprecipitates from a 0.5% DOC-1% Triton X-100 mitochondrial lysate incubated with normal serum γ -globulins (lane 2) or with antiserum 1 against beef heart complex I (lane 3). *b*, Immunoprecipitates from a 0.5% Triton X-100 mitochondrial lysate of HeLa cells labelled with ³⁵S-methionine for 2 h, then chased for 2 h (lane 5) or 18 h (lanes 7, 8 and 9), after incubation with antiserum 2 against beef heart complex I (lanes 5 and 8), or with anti-COII-C γ -globulins (lane 7), or with normal serum γ -globulins (lane 9). Lanes 1, 4, 6, pattern of mitochondrial translation products.

Methods. *a*, Two samples of mitochondrial suspension from HeLa cells labelled and chased as described in Fig. 2 legend (3×10^5 c.p.m.; 325 μ g protein; lanes 2, 3) were solubilized with 0.5% DOC for 30 min at 4°C and treated subsequently with 1% Triton X-100 as described previously²⁵; after centrifugation at 25,000 r.p.m. in a Spinco 65 rotor for 40 min, the supernatant was incubated with 150 μ g normal serum γ -globulin (lane 2) or 15 μ l antiserum 1 against beef heart complex I (lane 3). After 2 h incubation at 4°C and addition of 3 mg unlabelled mitochondrial proteins and SDS to 0.1%, the samples were mixed with 125 μ l Zysorbin suspension and further treated as described for the 0.5% Triton X-100-solubilized samples. *b*, Samples of mitochondria from HeLa cells labelled and chased as described in Fig. 2 legend (10^5 c.p.m., 110 μ g protein; lane 8), or chased for 2 h after the ³⁵S-methionine pulse instead of 18 h (2×10^5 c.p.m., 190 μ g protein; lane 5), were solubilized with 0.5% Triton X-100 and immunoprecipitated with 25 μ l (18 h chase) or 50 μ l (2 h chase) of antiserum 2 against beef heart complex I, as described in Fig. 2 legend. Equivalent portions of the mitochondrial lysate from 18-h chased cells were incubated with 250 μ g γ -globulins from anti-COII-C antiserum (lane 7) or from normal serum (lane 9). Exposure of both autoradiograms in *b* was for 20 days. In a repetition of the immunoprecipitation experiment with 2-h chased material, the normal serum control showed bands of COI, COII and COIII of equal intensity to those of the antiserum-precipitated sample.

experiment, the solubilized complex I had been exposed to 0.4–0.5% Triton X-100 during the immunoprecipitation and subsequent washing steps (see Fig. 4 legend). Similar results were obtained in another experiment involving exposure of complex I to 0.2% DOC in the absence of Triton X-100 throughout the entire procedure. More drastic conditions of detergent treatment, namely solubilization with 0.5% DOC in the presence of 0.5 M KCl and subsequent treatment with 1% Triton X-100, seem to disrupt the integrity of the complex containing the human URF products. These conditions have been shown previously to leave most of the bovine complex I intact, but to cause a loss of NADH-ubiquinone oxidoreductase activity, although not of NADH-K₃Fe(CN)₆ oxidoreductase activity²⁵. When the anti-complex I antiserum was tested on mitochondrial membranes treated as outlined above, only the URF4 and URF5 products were detected in the immunoprecipitate (Fig. 5a, lane 3); a minimal amount of URF2 and URF1 products was visible in the autoradiogram only after long exposure.

Another factor playing a considerable part in the immunoprecipitation pattern of the URF products are the labelling

conditions. When immunoprecipitation tests were performed on a Triton X-100 mitochondrial lysate from HeLa cells that had been subjected to a short (2 h) chase instead of an 18-h chase, the concentration of URF5 and 4 products precipitated was more than an order of magnitude lower and the amount of URF2 three- to fourfold lower than with material chased for 18 h (compare lanes 5 and 8, Fig. 5). This observation suggests that the incorporation of the newly synthesized URF products into complex I is slow. The experiments of Fig. 5b were performed with a different antiserum against bovine complex I (antiserum 2) from that used in the experiments described previously; this antiserum did not precipitate complex I from solubilized human kidney submitochondrial particles, although it reacted with four polypeptides in immunoblot assays. This antiserum, although less effective than antiserum 1, gave a cleaner immunoprecipitate from Triton X-100-solubilized mitochondria than antiserum 1 (compare Fig. 4, lane 6 with Fig. 5, lane 8).

Fraction enriched in six URF products

The results described above strongly suggest that the human respiratory-chain NADH dehydrogenase contains polypeptides encoded in six of the mtDNA URFs. To obtain independent evidence, a mixture of ^{35}S -methionine-labelled HeLa cell mitochondria and human heart mitochondria was subjected to the fractionation scheme which has been used to purify NADH-cytochrome *c* reductase (complex I-III of the respiratory chain) from beef heart mitochondria²⁸. The cytochrome *c* oxidase complex from human heart could not be fractionated in the same way as the complex derived from beef heart; as a result, COI, COII and COIII contaminated all fractions to various degrees. Despite this and the constraints imposed on the amount of unlabelled material usable for purification by the requirement for gel analysis and autoradiography, we obtained two fractions (P_5 and P_6 , Table 1) enriched in NADH- Q_1 and NADH- $\text{K}_3\text{Fe}(\text{CN})_6$ oxidoreductase activities. These fractions were essentially free of H^+ -ATPase complex, inferred from the almost complete absence of the ATPase 6 and URFA6L polypeptides (Fig. 6). Densitometric measurements showed that ~90% of the ATPase 6 and URFA6L polypeptides remained in the supernatant in the early fractionation step in which complexes I, II and III are precipitated. Fractions P_5 and P_6 were also free of complex II, assayed by succinate dehydrogenase activity. The two fractions were enriched greatly in the six URF products (compare lanes P_5 and P_6 with the marker lane M containing the starting material in Fig. 6). This enrichment could not be correlated with the presence of either cytochrome *b-c*₁ complex or complex II; the P_6 supernatant, which contained most of the cytochrome *b* polypeptide (estimated ~80% by densitometry) and almost all the succinate dehydrogenase activity of the P_5 + P_6 supernatant fractions, showed only a small amount of URF products (estimated to be 10–15%; Fig. 6). Similarly, the enrichment in URF products of P_5 and P_6 could not be attributed to the presence of cytochrome *c* oxidase in these fractions, because both biochemically purified²² and immunoprecipitated cytochrome *c* oxidase (P.M. *et al.*, in preparation) are totally devoid of URF products. Therefore, it seems that, among the five

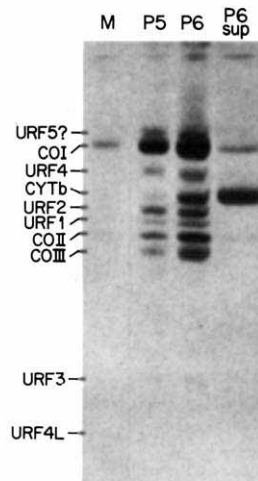


Fig. 6 Distribution of mitochondrial translation products among submitochondrial fractions. P_5 , P_6 and P_6 supernatant (P_6 sup) are as defined in Table 1. M, Starting material.

Methods. A mixture of mitochondria from HeLa cells labelled as described in Fig. 2 legend (1.6×10^7 c.p.m., 17 mg protein) and human heart mitochondria (200 mg protein) was subjected to a fractionation scheme for the isolation of NADH-cytochrome *c* reductase (complex I-III)²⁸. Equivalent amounts of protein (6.5 μg) of various fractions were run on an SDS/urea polyacrylamide gel.

enzymatic complexes of the oxidative phosphorylation system, the only one whose enrichment can be correlated with the enrichment of P_5 and P_6 in URF products is complex I. From densitometric measurements on the autoradiogram of a gel in which equal amounts of radioactivity of the various samples were analysed, P_5 was estimated to be enriched relative to the starting material ~7-fold in URF4 product and ~5-fold in URF2 and URF1 products; P_6 was enriched ~11-fold in URF4 product and ~7- and 11-fold, respectively, in URF2 and URF1 products. Therefore, the enrichment in URF products in P_5 and P_6 is similar to the enrichment in NADH- Q_1 and NADH- $\text{K}_3\text{Fe}(\text{CN})_6$ oxidoreductase activities. The deviations from perfect proportionality between NADH dehydrogenase activities and enrichment of URF products in different fractions results from an underestimation of enzyme activities in P_5 and P_6 , because of the decrease in solubility of the enzyme that occurs during purification.

Discussion

The conclusion that URF1, URF2, URF3, URF4, URF4L and probably URF5 encode components of the respiratory-chain NADH dehydrogenase is based on four lines of evidence: (1) The products of these URFs are co-precipitated from a Triton X-100 lysate of HeLa cell mitochondria by anti-peptide antibodies specific for individual URF products, especially by anti-URF4L, which suggests that they are components of a complex. (2) Antibodies raised against highly purified bovine complex I react weakly, though specifically, with two URF

Table 1 Enzyme activities of mitochondrial fractions

Fraction	NADH-ferricyanide oxidoreductase (μmol NADH oxidized per min per mg protein) ³⁵	NADH- Q_1 oxidoreductase (μmol NADH oxidized per min per mg protein) ³⁵	Succinate dehydrogenase (μmol succinate oxidized per min per mg protein) ³⁶
Mitochondria	2.7	0.04	0.02
P_5	11.0	0.14	ND
P_6	20.0	0.13	ND
P_6 supernatant	10.0	0.07	0.34

The P_5 fraction is equivalent to complex I-III of Hatefi and Stiggall²⁸, except that 6.4 ml of 50%-saturated NH_4OAc were added to the previous supernatant per 100 ml of supernatant. The P_6 fraction was obtained from the P_5 supernatant by addition of 6.4 ml of 50%-saturated NH_4OAc per 100 ml of supernatant and pelleting. ND, not detectable at the protein concentration used in the assays.

products in an SDS mitochondrial lysate, that is, with the product of URF4 and the putative product of URF5. (3) The antibodies against bovine complex I precipitate the same six URF products from a DOC or Triton X-100 mitochondrial lysate. (4) A submitochondrial fraction enriched in NADH-Q₁ and NADH-K₃Fe(CN)₆ oxidoreductase activities is also enriched in the products of the six URFs.

The URF4-, URF2- and URF3-specific anti-peptide antibodies were less effective than the URF4L-specific antibodies in co-precipitating the other URF products. This may result from a dissociation of the URF product(s) from the complex induced by a destabilizing effect of the antibodies, as has been reported for another mitochondrial complex²⁹, or to a poor accessibility of the peptide in the complex. The lower capacity of antibodies against native complex I to precipitate URF products from an SDS mitochondrial lysate than from a DOC or Triton X-100 lysate may be because of poor recognition of the SDS-denatured polypeptides by the antibodies, or the effect of SDS on antibody reactivity. Further, it is probable that antibodies against extramitochondrially synthesized components of complex I are also involved in precipitating the complex.

The results described above clearly indicate that the products of URF1, URF2, URF3, URF4, URF4L and the putative product of URF5 are associated closely with complex I. Because this association withstands a variety of detergent treatments, as well as the fractionation procedure for separation of the complexes of the oxidative phosphorylation system, the most plausible interpretation is that the URF products are themselves part of complex I. The enzymatic purity of the complex I preparations used to make the antisera²⁴ and the evidence indicating that the polypeptides found in complex I preparations are indeed true constituents of the enzyme²⁵ strongly support this interpretation. It is possible that at least some of the URF products are part of the shell of hydrophobic proteins surrounding the catalytic subunits^{20,21}. The estimated *M_r*s of the URF products discussed here²³, 51,000 (URF5), 39,000–36,000 (URF4), 25,000 (URF2), 24,000 (URF1), 6,000 (URF3) and 3,500 (URF4L), are in reasonable agreement with the reported *M_r*s for some of the proteins of the insoluble fraction and of the iron-protein fragment of the bovine complex I (ref. 20). We propose that the designations of URF1, URF2, URF3, URF4, URF4L and URF5 be changed to ND (for NADH dehydrogenase) 1, ND2, ND3, ND4, ND4L and ND5, respectively.

In previous work, Hare and Hodges¹⁹ failed to detect any mitochondrially synthesized polypeptide in complex I solubilized from a mixture of unlabelled rat liver mitochondria and labelled rat hepatoma mitochondria and precipitated by antibodies against beef heart complex I. But the labelling conditions used by those authors (2-h exposure of the cells to ³⁵S-methionine without subsequent chase) would allow only very little incorporation of the mitochondrially synthesized, ³⁵S-methionine-labelled subunits into complex I. Further, the authors solubilized complex I from submitochondrial particles with 0.5% DOC and

1% Triton X-100, conditions that we have observed to cause the almost total loss of the four smallest URF products from the HeLa cell immunoprecipitated complex I.

In a more recent investigation³⁰, the analysis of the mitochondrial translation products and enzyme complexes in a Chinese hamster lung cell mutant, which shows a great decrease in NADH dehydrogenase and cytochrome *c* oxidase activities resulting from an apparently single nuclear mutation, led to the suggestion that complex I contains one or more mtDNA-encoded components^{31,32}. The present work provides strong direct evidence for this suggestion.

Functional and evolutionary implications

Yeast mtDNA does not seem to contain any gene homologous to the animal mtDNA URFs that encode components of NADH-ubiquinone oxidoreductase. The NADH dehydrogenase region of the respiratory chain, however, exhibits functional and structural differences in *Saccharomyces* strains compared with the mammalian counterpart; in particular, it lacks site 1 energy coupling³³. These differences could conceivably result from or be correlated with the absence of some or all of the polypeptides homologous to the animal mtDNA URF products from the yeast complex. On the other hand, it is quite possible that some or all of these polypeptides are encoded in the nucleus and imported from the cytoplasm in yeast. Note that, in contrast to *Saccharomyces* mtDNA, *A. nidulans* mtDNA contains four reading frames that have a convincing amino-acid sequence homology to human URF1, URF3, URF4 and URF5 (ref. 9); a gene homologous to URF1 has been found in *N. crassa* and in *Zea mays*⁹. Genes having a small but significant amino-acid sequence homology to human URF1, URF4 and URF5 and to human URF4 and URF5, respectively, also occur in the kinetoplast maxicircle DNA of *Trypanosoma brucei*¹⁰ and of *Leishmania tarentolae*¹¹.

It will be of interest to see whether the variation in the complement of mtDNA-encoded subunits in the respiratory-chain NADH dehydrogenase of different organisms is correlated with functional differences. The finding of a variability in genetic control of NADH-ubiquinone oxidoreductase in different organisms extends similar observations concerning the genetic determination of the H⁺-ATPase complex³⁴ and has interesting implications for the evolution of the mitochondrial genome. The evolutionary processes that have led to the segregation of mitochondrial and nuclear genes involved in mitochondrialogenesis apparently have followed different paths and progressed to a different extent in different organisms, producing the variety of patterns observed today.

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- Anderson, S. *et al.* *Nature* **290**, 457–465 (1981).
- Bibb, M. J., Van Etten, R. A., Wright, C. T., Walberg, M. W. & Clayton, D. A. *Cell* **26**, 167–180 (1981).
- Anderson, S. *et al.* *J. molec. Biol.* **156**, 683–717 (1982).
- Pepe, G. *et al.* *Biochem. Int.* **6**, 553–563 (1983).
- Clary, D. O. & Wolstenholme, D. R. *Nucleic Acids Res.* **11**, 6859–6872 (1983).
- Clary, D. O., Wahlleitner, J. A. & Wolstenholme, D. R. *Nucleic Acids Res.* **12**, 3747–3762 (1984).
- DeBruijn, M. H. L. *Nature* **304**, 234–241 (1983).
- Davies, R. W. *et al.* in *Mitochondrial Genes* (eds Slonimski, P., Borst, P. & Attardi, G.) 405–410 (Cold Spring Harbor Laboratory, New York, 1982).
- Scazzocchio, C., Brown, T. A., Waring, R. B., Ray, J. A. & Davies, R. W. in *Mitochondria 1983. Nucleo-Mitochondrial Interactions* (eds Schwenen, R. J., Wolf, K. & Kaudewitz, F.) 303–312 (de Gruyter, Berlin, 1983).
- Hensgens, L. A. M. *et al.* *Nucleic Acids Res.* **12**, 7327–7344 (1984).
- De La Cruz, V. F., Neckelmann, N. & Simpson, L. *J. biol. Chem.* **259**, 15136–15147 (1984).
- Mariottini, P. *et al.* *Cell* **32**, 1269–1277 (1983).
- Chomyn, A. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **80**, 5535–5539 (1983).
- Macreadie, I. G. *et al.* *Nucleic Acids Res.* **11**, 4435–4451 (1983).
- Attardi, G. *Int. Rev. Cytol. Suppl.* **17** (in the press).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105–132 (1982).
- Hatefi, Y., Haavik, A. G. & Griffiths, D. E. *J. biol. Chem.* **237**, 1676–1680 (1962).
- Hatefi, Y. & Stiggall, D. L. *Enzymes* **13**, 175–297 (1976).

- Hare, J. F. & Hodges, R. *Biochem. biophys. Res. Commun.* **105**, 1250–1256 (1982).
- Ragan, C. I. in *Subcellular Biochemistry* Vol. 7 (ed. Roodyn, D. B.) 267–307 (Plenum, New York, 1980).
- Hatefi, Y., Ragan, C. I. & Galante, Y. M. in *The Enzymes of Biological Membranes* Vol. 4 (ed. Martonosi, A.) 1–70 (Plenum, New York, 1984).
- Hare, J. F., Ching, E. & Attardi, G. *Biochemistry* **19**, 2023–2030 (1980).
- Ching, E. & Attardi, G. *Biochemistry* **21**, 3188–3195 (1982).
- Heron, C., Smith, S. & Ragan, C. I. *Biochem. J.* **181**, 435–443 (1979).
- Smith, S. & Ragan, C. I. *Biochem. J.* **185**, 315–326 (1980).
- Smith, S., Cottingham, I. R. & Ragan, C. I. *FEBS Lett.* **110**, 279–282 (1980).
- Storrie, B. & Attardi, G. *J. molec. Biol.* **71**, 177–199 (1972).
- Hatefi, Y. & Stiggall, D. L. *Meth. Enzym.* **31**, 5–10 (1978).
- Marzuki, S. *et al.* in *Mitochondria 1983. Nucleo-Mitochondrial Interactions* (eds Schwenen, R. J., Wolf, K. & Kaudewitz, F.) 535–549 (de Gruyter, Berlin, 1983).
- Malczewski, R. M. & Whitfield, C. D. *J. biol. Chem.* **259**, 11103–11113 (1984).
- Chu, E. H. Y., Sun, N. C. & Chang, C. C. *Proc. natn. Acad. Sci. U.S.A.* **69**, 3459–3463 (1972).
- Malczewski, R. M. & Whitfield, C. D. *J. biol. Chem.* **257**, 8137–8142 (1982).
- Ohnishi, T. *Biochim. biophys. Acta* **301**, 105–128 (1973).
- Attardi, G., Chomyn, A. & Mariottini, P. in *H⁺-ATP Synthase: Structure, Function, Regulation* (eds Papa, S., Altendorf, K., Ernster, L. & Packer, L.) 25–40 (ICSU and Adriaatica Editrice Bari).
- Galante, Y. M. & Hatefi, Y. *Archs Biochem. Biophys.* **192**, 559–568 (1979).
- Hanstein, W. G., Davis, K. A., Ghalambor, M. A. & Hatefi, Y. *Biochemistry* **10**, 2517–2524 (1971).

Identification and comparison of two sequence elements that confer cell-type specific transcription in yeast

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The MAT α 2 protein of budding yeast represses a set of genes; if the MAT α 1 protein is also present, a further set of genes is repressed. DNA sequence comparisons reveal a 20-base pair 'operator' sequence that is present in genes repressed by α 1/ α 2. A related, but distinct, sequence is found in genes repressed by α 2 alone.

CELL type in the yeast *Saccharomyces cerevisiae* is determined by the allele (*MAT α* or *MAT α 2*) that occupies the *MAT* locus¹. Mackay and Manney^{2,3} proposed that *MAT* is a regulatory locus controlling the expression of several unlinked genes required for mating or sporulation. A physical comparison of *MAT α* and *MAT α 2*^{4,5} has revealed that each allele produces two transcripts, (α 1, α 2) or (α 1, α 2). Strathern *et al.*⁶ proposed that the *MAT α 1* protein is a positive regulator of some of the genes required for α mating, whereas *MAT α 2* is a negative regulator of some of those required for a mating. In a/α diploids, *MAT α 1* and *MAT α 2* act together as a negative regulator of genes not required in diploids, including *MAT α 1* (Fig. 1; see ref. 7 for a recent review).

In several cases this regulation seems to be exerted at the level of transcription; for example, α 2 function is required for the repression of transcription of the a -specific gene *STE6* (ref. 8) and is thought to act similarly on other genes involved in producing the a mating phenotype, such as *STE2*, *BAR1* and *MF α 1* (V.L.M., unpublished results; A. Brake, personal communication). Once an α cell has mated with an a cell to produce an a/α diploid, the function of α 2 becomes modified. As well as its function in α cells, it now acts in conjunction with the *MAT α 1* gene product to repress an additional set of genes. Therefore, these genes are repressed only if both *MAT α 1* and *MAT α 2* are expressed. Such 'haploid-specific' genes include *MAT α 1* (refs 4, 9), *HO* (ref. 10) and *STE5* (J. Thorner, personal communication and V.L.M., unpublished results). Thus, the α 2 gene product represses one set of genes in α cells but an additional set of genes in a/α diploids.

Both *MAT α 1* and *MAT α 2* show protein sequence homology with the 'homoeo domain'¹¹, a highly conserved protein sequence found in gene products involved in selecting developmental fates in *Drosophila melanogaster*¹²⁻¹⁴. It has also been located in genes of unknown function in other phyla, including vertebrates^{15,16}. In the case of *MAT α 1*, part of the homoeo domain is strikingly similar to the DNA binding domain of bacterial repressors¹⁷, suggesting that *MAT α 1* encodes a repressor protein. There is more direct evidence that the *MAT α 2* gene encodes a DNA binding protein. An α 2- β -galactosidase fusion protein binds specifically to the 5'-flanking DNA of *STE2* and *STE6* (A. Johnson, personal communication).

Here we have identified the DNA sequences recognized by the α 2 and the α 1/ α 2 repression systems, first by finding candidates on the basis of DNA sequence comparisons, and second by demonstrating that the proposed sequences are sufficient to confer the appropriate control on an unrelated promoter. Our proposed α 2 and α 1/ α 2 control sequences are different but related; we discuss the means by which the two sets of targets (α 2 against α 1/ α 2) are distinguished.

α 1/ α 2 Control of the *HO* gene

One of the haploid-specific genes repressed by the action of α 1 and α 2 is the *HO* gene¹⁰, which is responsible for initiating

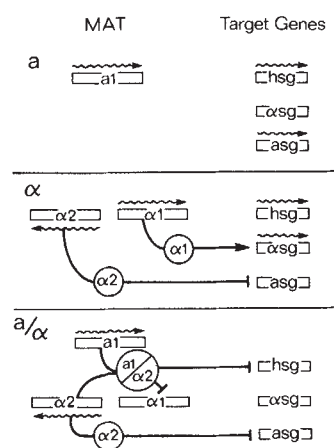


Fig. 1 Mating type control. Left, *MAT* genes: right, unlinked genes which are the targets for their action. Wavy line, gene expression; heavy line, effect of a gene product on the expression of other genes. In an a cell, a -specific genes (*asg*) and haploid-specific genes (*hsg*) are expressed constitutively. In an α cell, the *asg* are repressed by the action of the α 2 gene product, while α -specific genes (α sg) are activated by the α 1 gene product⁶. When these two cell types mate to produce an a/α diploid, the α 1 and α 2 gene products together repress the expression of the haploid-specific genes, including *MAT α 1*.

mating type switching (ref. 18 and F. Heffron, personal communication). We have therefore investigated the α 1/ α 2 control of this gene.

Two lines of evidence suggest that the sequences that confer α 1/ α 2 repression on *HO* transcription are present in multiple copies distributed widely in the 5'-flanking DNA. The first comes from the deletion analysis by Jensen¹⁹ of plasmids carrying an *HO-lacZ* fusion. A deletion removing all but 296 base pairs (bp) of the DNA flanking the 5' end of the gene (as measured from the AUG) is fully constitutive, whereas one removing all but 762 bp is fully repressed by the a/α state. This suggests that α 1/ α 2 control sequences are 5' to the gene and that they must lie between -296 and -762 from the AUG (Fig. 2). Surprisingly, a deletion (no. 229.102) which removes all the DNA between -172 and -928 is not only transcribed efficiently, but also is fully repressed by the a/α state when transplanted at the *HO* locus²⁰. This suggests that there must be at least two copies of the putative α 1/ α 2 control element, one between -296 and -762, and another either upstream of -928 or in the 3'-flanking sequences.

The distribution of the α 1/ α 2 control elements has also been studied by insertions of the yeast *TRP1* gene at various positions in the *HO* locus. The 850-bp *EcoRI/BglII* fragment of *TRP1*

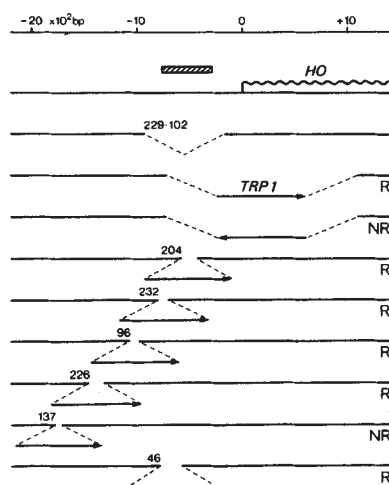


Fig. 2 Redundant a/α control of the *HO* gene. Hatched box, the region defined as important by Jensen¹⁹; wavy line, ORF of the *HO* gene. The extent of deletion 229.102 is shown. Insertions of the *TRP1* gene into various deletions are also shown; R indicates that in a particular insertion, the *TRP1* gene is repressed in a/α cells; NR indicates that the *TRP1* gene is expressed constitutively. The arrow on the *TRP1* gene indicates the direction of transcription.

Methods. Disruptions of the *HO* gene were constructed and transplanted²⁰ into the chromosomal *HO* locus of strain K765 (*HMLa MATa HMRa ho::SUP4-o trp1-1 ade2-1 can1-100 his3 leu2-3, 112 ura3*). All insertions fully complemented the *trp1-1* mutation of K765. To test the a/α repression, the transformants were crossed to strain K822 (*HMLa MATa HMRa ho trp1-1 ade2 can1-100 his4 leu2 ura3*) and the *TRP1* phenotype of the resulting diploids was scored. In the case of *TRP1* replacing the DNA from -718 to +1,096 (in the same orientation as the *HO* coding sequence), it was shown that the insertion complemented *trp1-1* in a/α and α/α cells, but not in isogenic a/α cells.

contains all of the coding sequences of the gene encoding (5-P-ribosyl)-anthranilate isomerase but few of its 5'-flanking sequences (only out to -102 from the AUG). Because of its incomplete promoter, the level of expression of this fragment is prone to position effects²¹. Therefore, we have used it as a probe for the regulatory properties of the DNA flanking *HO* by analysing the *TRP1* phenotype in a , α or a/α *trp1*⁻ cells containing various insertions of *TRP1* in *HO*.

Several *TRP1/HO* insertions (see Fig. 2) have been transferred to the chromosomal *HO* locus using the *SUP4-o* transplacement technique²⁰. All the constructions described in Fig. 2 lead to a full *TRP1* phenotype in the parent a strain. Many of the insertions, however, lose the ability to confer tryptophan prototrophy when an a/α diploid is formed. We have used this specific loss of tryptophan prototrophy as a qualitative measure of the repression of the *TRP1* gene by a/α control. For example, the *TRP1* gene is fully repressed by the a/α state when inserted (in the same orientation as the *HO* coding sequence) as a replacement for the *HO* DNA from -718 to +1,096. In contrast, the gene is constitutive (that is, can confer a *TRP1* phenotype to a , α and a/α cells) when inserted in the opposite orientation. The simplest interpretation of this result is that *HO* sequences to the left of position -718 can confer a/α repression on the *TRP1* gene and, moreover, they will only do so if joined to its 5' end. The *TRP1* gene is also regulated when placed (in the same orientation as the *HO* coding sequence) in a number of positions within the interval -300 to -1,450 (see Fig. 2). In contrast, it is not repressed (at least qualitatively) in deletion 137 (-1,737 to -1,777).

These results with *TRP1/HO* fusions, taken together with the phenotype of deletion 229.1002 and the results of Jensen¹⁹, suggest that there are several a/α control elements in the interval between -400 and -1,400, and that the 5' end of the *TRP1* gene

must be near one such element to be regulated. Alternatively, there may be fewer elements (although there must be at least two) effective over long distances. Interestingly, an insertion of *TRP1* in the opposite orientation (that is, 5' end at the 3' *HO* side) in deletion 46 (-582 to -791) is also under a/α control. This is consistent with the data of Jensen¹⁹ showing that a/α control elements exist in the -296 to -762 interval and suggests that there is one such element to the right of position -582.

In summary, the a/α control of the *HO* gene is redundant, containing two or more control sites within the region -1,777 to -296.

Search for a repeated sequence

In the light of the results described above, the *HO* regulatory region was searched by computer to see whether it contained a repeated sequence motif that satisfied the above criteria. This revealed a sequence motif (T/C)(A/G)TGTTNN(A/T)-NANNTACATCA (Table 1) that is repeated at least 10 times in the 5'-flanking region of the *HO* gene. From the number of occurrences of this motif, its appearance cannot be a chance event; therefore, it must be fulfilling some function. If it is the sequence responsible for $a1/a2$ control, then we would expect to find it in front of other genes that are repressed by $a1/a2$. Therefore, we have searched the 5'-flanking regions of two genes that are also repressed in the diploid, *MATa1* and *STE5*, for homology with the consensus sequence. At *MATa1* there is one occurrence of this sequence (one ' $a1/a2$ element'), which lies in the region defined as necessary for $a1/a2$ control²². At *STE5* (J. Schultz and J. Thorner, personal communication), there are

Table 1 Examples of the $a1/a2$ element

SEQUENCE	SOURCE	"SCORE"
CAATGTAGAAAAGTACATCA	MATa1	-13.5
GCTTGTTAATTTACACATCA	STE5 -196	-15.0
TCATGTACTTTTCTGCATCA	STE5 -179	-13.0
CCGCGTTAAAACCTACATCA	HO -1752	-14.4
TTATGTTAAAAGTTACATCC	HO -1391	-13.2
GCCTGCGATGAGATACATCA	HO -1328	-17.2
TAGAGTGAAGAACATCG	HO -1208*	-18.5
TCATGTATTCATTCACATCA	HO -736*	-10.6
ACATGTCCTCACTGCATCA	HO -669*	-14.7
TCGTGATTTACTTACATCA	HO -576*	-10.0
TCATGTTATTATTACATCA	HO -411	-8.7
TCATGTCCACATTACATCA	HO -371	-12.8
CGGTTAGAACGCTTCATCA	HO -150*	-18.1
CONSENSUS: TC _A GTGTTNN _A NANNTACATCA		

The sequences of the $a1/a2$ elements that we have found in the *MATa1*, *STE5* and *HO* genes are shown. For the elements marked with an asterisk, the orientation of the element has been reversed (taking the complementary strand) to give a closer fit to the consensus sequence. The 'score' shown is a measure of the fit of each element to the consensus sequence²⁶; the larger the negative number, the poorer the fit. Thus, the best fit to the consensus sequence is the *HO* -411 element used in the sufficiency experiment (Fig. 3, insert B). An approximate consensus sequence is shown at the foot of the table. Note that this is an imperfect inverted repeat. Sequence information: *MATa1* from ref. 34, *STE5* from J. Schultz and J. Thorner (personal communication), and *HO* from M. Squire and M. Smith (personal communication). The 5'-flanking sequence of the *HO* gene (1,950 bp) was compared with itself and with its complementary strand, using *DIAGON*²⁵; this revealed a number of examples of the putative $a1/a2$ element. The *HO* sequence was compared with the *MATa1* intergenic region, revealing the same sequence at *MATa1*: these sequences were aligned to produce a frequency matrix (using *GETFRQ*) and the *HO* sequence was re-analysed using the *ANALYSEQ* program suite²⁶. This revealed a total of 10 examples of this motif in the *HO* gene and two examples in 705 bp of 5'-flanking sequence at *STE5*. A frequency matrix was then constructed including all known instances of the motif; the aligned sequences are shown in the table. The frequency matrix used to search the EMBL library was derived from these examples, except that the *MATa1* sequence was included three times to give it greater weight. We have re-evaluated all the occurrences we have found, testing their score against a frequency matrix that lacks this arbitrary weighting. Thus, all the 'scores' described here refer to a frequency matrix derived from the set of examples shown in the table.

two such *a1/a2* elements in tandem, ~200 bp upstream from the AUG (Table 1).

The computer search of the *HO* gene also revealed another repeated sequence, CACGAAAA. All occurrences of this sequence, however, are contained in the endpoints of the 229.102 deletion, which still retains *a1/a2* control. This CACGAAA motif seems to be involved in the cell-cycle-dependent transcription of the *HO* gene²³.

Control by the repeated sequence

We have asked whether this sequence ((T/C)C(A/G)-TGTNN(A/T)NANNTACATCA) is able to bring an unrelated promoter under cell-type control. To do this we have used the *CYC1* promoter fused to the *Escherichia coli* β -galactosidase gene²⁴. The *CYC1* promoter consists of a TATA box and an upstream element required for efficient transcription, the upstream activating site (UAS). This UAS is sensitive to carbon source, being partially repressed when the yeast are grown on glucose-containing media²⁵. We have investigated the effect of inserting an *a1/a2* element into the region between the UAS and the TATA box. We took two different examples of the *a1/a2* element from the *HO* gene and inserted them into a *XhoI* site in the *CYC1* promoter, then asked how these inserts affected the activity of the promoter in different cell types. We find that the parent plasmid and the two insert-containing plasmids all direct the production of similar levels of β -galactosidase activity in isogenic *a*, α and a^- cells (when tested on indicator plates; data not shown). If we take an α cell carrying the plasmid, we can either mate it to an *a* cell or an a^- cell to produce two different diploids. One, the *a/a*, is a normal diploid and will repress its haploid-specific genes. The other, the *a^-/a* cell, will not repress its haploid-specific genes because *MATa1* is absent. When the β -galactosidase activity of these strains is measured, we find that the parent plasmid is not affected by the difference in mating type (Fig. 3). The plasmids containing an *a1/a2* element, however, show a dramatic reduction in the level of β -galactosidase activity in the *a/a* diploid compared with the *a^-/a* diploid. This implies that a *CYC1* promoter containing an *a1/a2* element is sensitive to *a/a* control.

This result could arise if the insertion of the *a1/a2* element inactivated the *CYC1* promoter and simultaneously generated a novel *a/a*-sensitive promoter to replace it. To demonstrate that the constructions are still being 'driven' by the *CYC1* UAS, we have tested the carbon source dependence of the plasmids. When grown on raffinose (instead of glucose) the parent plasmid and the two insert-containing plasmids all produce a similar, approximately threefold increase in β -galactosidase (Fig. 3). Therefore, the activity of the *CYC1* UAS is blocked in *a/a* diploids by the presence of an *a1/a2* element between the UAS and the TATA box.

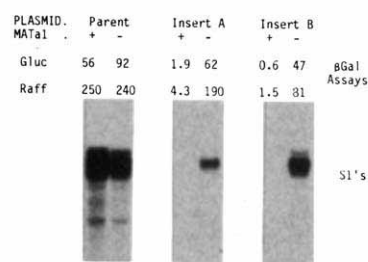
Figure 3 also shows that in α cells the insert-containing plasmids produce transcripts identical to those produced by the parent plasmid, but in *a/a* cells no transcript is seen. Therefore, the control is operating at the level of transcription. We conclude that our *a1/a2* element is sufficient to confer cell-type-specific expression on a gene.

Search of other genes

Because the *a1/a2* element seems to be sufficient to confer cell-type-specific repression, we would not expect to find good matches to this sequence in genes that are not under *a/a* control. We have searched the European Molecular Biology Laboratory (EMBL) nucleotide sequence library to see whether there are other genes containing this sequence motif, using the ANALY-SEQ program²⁶. A matrix was constructed containing the frequencies at which each nucleotide T, C, G or A occurs at each position in the *a1/a2* elements. This matrix was then used to scan DNA sequences to find regions showing a good fit to the consensus sequence.

Some caution must be exercised in interpreting the results of such a search, because the 'goodness-of-fit' observed refers to the consensus sequence that we have constructed and does not

a EFFECT OF THE *a1/a2* ELEMENT ON THE *CYC1* PROMOTER



b

Parent
(= UAS)
CCGATGGCAATCTCGAGATCCACGAAAAATGATGTGAATCAATACATGAAGATTCTAGATCTCGAGCAGATCCGCC

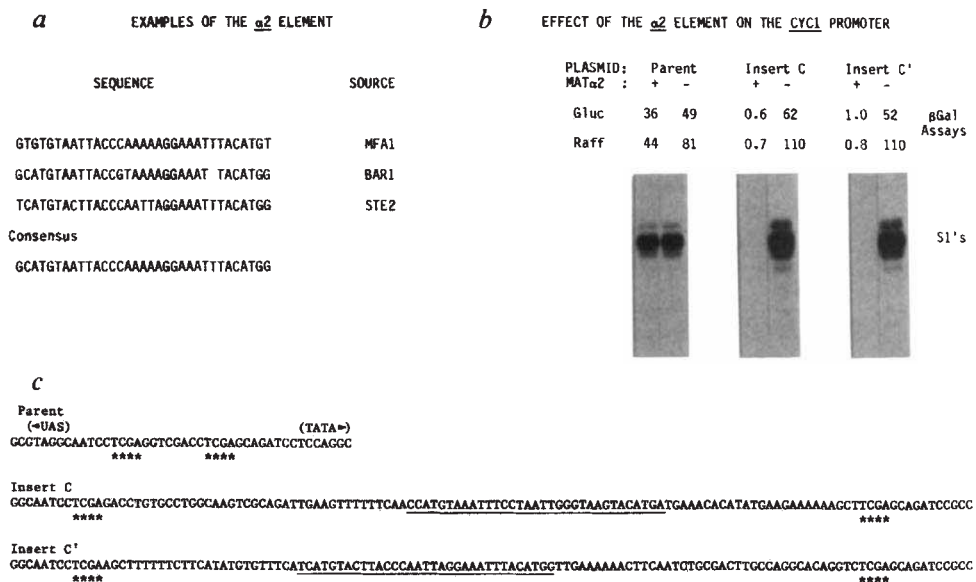
(TATA =)
Insert A
GGCAATCTCGAGATCCACGAAAAATGATGTGAATCAATACATGAAGATTCTAGATCTCGAGCAGATCCGCC

Insert B
GGCAATCTCGAGATCCACGAAAAATGATGTGAATCAATACATGAAGATTCTAGATCTCGAGCAGATCCGCC

Fig. 3 The *a1/a2* element is sufficient to confer *a/a* control. Two different examples of the *a1/a2* element from the *HO* gene were inserted between the UAS and the TATA box of the *CYC1* promoter driving the β -galactosidase gene. These plasmids were used to transform an *a/a* diploid and the isogenic *a^-/a* diploid. **a**, Levels of β -galactosidase (β -Gal) produced in exponentially growing cells are shown, both for raffinose and for glucose as carbon source. Levels of RNA produced by the plasmids were measured by the protection of radiolabelled DNA from *S*₁ nuclease digestion. **b**, Sequences of the constructions with the *a1/a2* elements underlined. Insert A is *HO* -736; insert B, *HO* -411 (Table 1). Asterisks denote the limits of the inserted fragments.

Methods. DNAs used: Plasmid pHO DNA source was a 4.3-kb *Sau3A* partial fragment and the *XhoI* linker mutant 204 derived from it²⁰. The *CYC1*- β -galactosidase fusion was pLGA312 (a gift of L. Guarente)³⁶. The vector for sequencing was M13 mp10 (ref. 37). Cloning of the *a1/a2* elements into the *CYC1* promoter. The *HO* plasmid was digested with *Bam*HI and *Bgl*II, filled in and blunt-end ligated into the filled-in *XhoI* site of pLGA-312 (insert A)³⁶. *XhoI*-linker mutant H204 was digested with *XhoI* and *NruI* and ligated into the *XhoI* site of pLGA312 (insert B). Recombinants were identified by colony hybridization³⁸ and their identity confirmed by subcloning and sequencing. The *SmaI*/*Bam*HI fragment of pLGA-312 carrying the *a1/a2* element insert was cloned into *Hinc*II/*Bam*HI-cut M13mp10. M13 carrying the relevant insert were identified by plaque hybridization and sequenced by the dideoxy-technique. β -Galactosidase assays were performed using the technique of Miller³⁹. Cells were collected by centrifugation, resuspended in Z buffer and permeabilized with *CHCl*₃/SDS. After the reaction was stopped with base, cell debris was removed by centrifugation before measuring the *A*₄₂₀. Units were measured as $1,000 \times A_{420} / A_{660} \times \text{time (min)}$. RNA samples were taken from cells growing exponentially on glucose and the level of transcription from the plasmid measured as described previously⁴⁰. The copy number of the plasmids did not vary. The DNA probe used is the *XhoI*/*Hinc*II fragment of pLGA-312 cloned into *Sall*/*SmaI*-cut M13mp9 (ref. 37). For the β -galactosidase assays the *MATa* strain used was K822 (Fig. 2); this was mated with either W303-1A (*HMLa MATa HMRa ade2-1 can1-100 his3-11, 15 leu2-3 trp1-1 ura3*) or its derivative RS3 (*HMLa mat::LEU2 hmr::TRP1 ade2-1 can1-100 his3-11, 15 leu2-3 trp1-1 ura3*). The *mat::LEU2* construction is described in ref. 21, and the *hmr::TRP1* construction was the kind gift of A. Brand (in preparation). These were transplaced into the yeast chromosome⁴¹. For the RNA measurements, the strains used were W303-1B (*HMLa MATa HMRa ade2-1 can1-100 his3-11, 15 leu2-3 trp1-1 ura3*) and Y65, which is isogenic, except that it carries a deletion of *HMR*_E that causes expression of *HMRa1* (A. Brand, manuscript in preparation.)

Fig. 4 Sequence of the $\alpha 2$ element and its effect on the *CYC1* promoter. **a**, Sequences of the $\alpha 2$ elements at the *Mfa1*, *Bar1* and *Ste2* genes. Note that the consensus sequence is an approximate inverted repeat. Sequence information is from A. Brake (personal communication), V.L.M. (unpublished results) and this work, respectively. **b**, Effect of the $\alpha 2$ element on the *CYC1* promoter. A 97-bp fragment of the *STE2* gene carrying an $\alpha 2$ element was inserted in both orientations into the *CYC1* promoter. Here we show the levels of β -galactosidase produced by these constructs in isogenic $\alpha 1^-$ and $\alpha 1^- \alpha 2^-$ cells, both for raffinose and for glucose as carbon source. Levels of RNA produced were measured by the protection of radio-labelled DNA from *S*₁ nuclease digestion. **c**, Sequences of the constructions with the $\alpha 2$ element underlined. Asterisks denote the limits of the inserted fragments.



Methods. The sequences of the 5'-flanking region of the *BAR1* (690 bp) and *Mfa1* (628 bp) genes were compared with each other, and with the 400-bp *HindIII* fragment of *STE2* using DIAGON³⁵. This revealed the sequences shown here. Sequencing *STE2*:pZV37 (which carries a *STE2*-complementing *PstI*/*EcoRI* fragment in pUC; V.L.M.) was digested with *HindIII* and ligated with *HindIII*-cut M13mp10. The sequence was then derived from each end by the dideoxy technique⁴². Inserting a fragment of *STE2* into the *CYC1* promoter. The plasmid carrying the *STE2* gene (pZV37) was digested with *HindIII* and *AvaII* and blunt-end ligated into *XhoI*-cut pLGA312. Recombinants were identified by colony hybridization and their identity confirmed by subcloning and sequencing. The *SmaI*/*BamHI* fragment of pLGA-312 carrying the $\alpha 2$ element insert was cloned into *HincII*/*BamHI*-cut M13mp10. These recombinants were sequenced. β -Galactosidase assays and RNA measurements were performed as in Fig. 3. The strains used were RS3 (Fig. 3, which is $\alpha 1^- \alpha 2^-$) and M48 (*HMLa MATa2 mata1::LEU2 hmr::TRP1 ade2-1 can1-100 his3-11, 15 leu2-3 trp1-1 ura3*) (this work). The *MATa2 mata1::LEU2* construction was the gift of L. Breeden (manuscript in preparation).

necessarily reflect exactly what is important for α/α repression *in vivo*.

We have searched 61 kilobases (kb) of yeast DNA sequence in the EMBL nucleotide sequence library and find only one significant match within the 5'-flanking region of any gene. The *Mfa1* gene²⁷ has the sequence CCATGTGAAAAGATGCATCT (which would score -15.5 in Table 1) at -230 bp from the AUG. *Mfa1* encodes the mating pheromone of the α cell and is an α -specific gene whose transcription is dependent on the *MATa1* gene product⁶ (Fig. 1). *Mfa1* is not expressed in α/α diploids, partly because *MATa1* is absent. There must, however, be a second α/α -dependent control as *Mfa1* is still not expressed in α/α diploids when *MATa1* is expressed constitutively²². Our finding that *Mfa1* contains a possible $\alpha 1/\alpha 2$ control element suggests that this second control is exerted directly by the $\alpha 1/\alpha 2$ repressor.

We also find several matches that occur in the open reading frames (ORFs) of genes. We believe that these occurrences of the $\alpha 1/\alpha 2$ element do not affect transcription, as transcription is not altered if the $\alpha 1/\alpha 2$ element is inserted into the ORF, rather than the promoter, of the *CYC1*- β -galactosidase gene fusion (data not shown). If the matches to the $\alpha 1/\alpha 2$ element found in ORFs have no biological effect, then they give a measure of the significance of the matches shown in Table 1. Our search revealed 10 examples of putative $\alpha 1/\alpha 2$ elements that would score -16.2 or better in Table 1; therefore, we conclude that such good fits to the consensus sequence should only occur at random once every 6 kb.

$\alpha 2$ -Mediated repression

We have shown that the sequence motif (T/C)C(A/G)TGTTNN-(A/T)NANNTACATCA is sufficient to confer $\alpha 1/\alpha 2$ -mediated repression. We know from the biology of yeast mating type that genes carrying this sequence (the haploid-specific genes) are not repressed by $\alpha 2$ alone (Fig. 1); $\alpha 2$ -mediated repression must involve a different, perhaps related, DNA sequence. To

address this question, we have analysed the DNA sequences of the 5'-flanking regions of three genes that are repressed by $\alpha 2$ alone, looking for sequences specific to these genes which are related to (but different from) the $\alpha 1/\alpha 2$ consensus. Using the DIAGON program of R. Staden³⁵, we have found that *Mfa1*, *Bar1* and *Ste2* all have a 30-bp sequence in common (Fig. 4a). Although in this instance we have only three examples of the sequence, it is clear from the extent of conservation that this sequence has some function. A. Johnson, K. Wilson and I. Herskowitz (personal communication) have also noted this homology.

Two lines of evidence suggest that this 30-bp sequence is involved in $\alpha 2$ control; first, an $\alpha 2$ - β -galactosidase fusion protein binds specifically to this DNA sequence (A. Johnson, personal communication). Second, we have shown that a 97-bp fragment containing the putative $\alpha 2$ element from the *STE2* gene can confer $\alpha 2$ control on the *CYC1* promoter. This has been done by inserting the 97-bp fragment between the UAS and the TATA box of the *CYC1* promoter. We then measured the levels of β -galactosidase in isogenic $\alpha 1^-$ and $\alpha 1^- \alpha 2^-$ strains; the only genotypic difference between these strains is that the former produces $\alpha 2$ but the latter does not. We find that the plasmid directs β -galactosidase production in the $\alpha 1^- \alpha 2^-$ cell, but not in the $\alpha 1^-$ cell. Further, the constructions are still under the same carbon source dependence as the parent plasmid, demonstrating that they are being driven by the *CYC1* promoter (Fig. 4b). Figure 4b also shows that the control is exerted at the level of transcription.

Thus, the 97-bp fragment can bring the *CYC1* promoter under $\alpha 2$ control. Because of the size of this fragment, we cannot be certain whether our consensus sequence alone is responsible for the regulation, or whether some other sequence on the fragment is involved. We believe that our consensus sequence is responsible because it is the most dramatic homology between the *Mfa1*, *Bar1* and *Ste2* sequences, and because it has a sequence related to that of the $\alpha 1/\alpha 2$ element (see below).

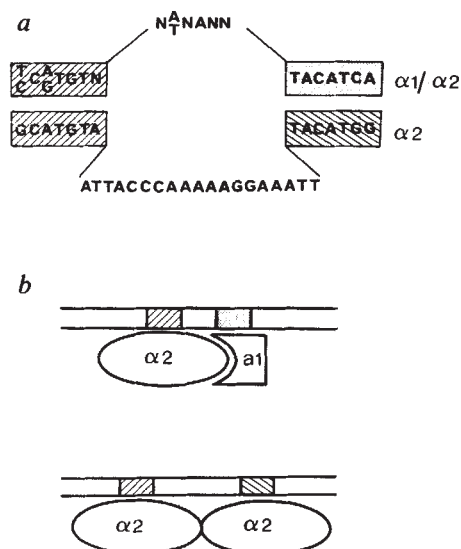


Fig. 5 *a*, Comparison of the $a1/a2$ and $a2$ elements. The $a1/a2$ element (top) is shown aligned with the $a2$ element (below) with the common sequence motifs boxed. The TACATG motif is hatched, and the TACATCA motif is stippled. *b*, The combinatorial aspect of mating type control. The $a2$ element (bottom) consists of two $a2$ half-sites correctly spaced to accommodate the $a2$ dimer. The $a1/a2$ element (above), in contrast, consists of an $a1$ and an $a2$ half-site spaced correctly for the 'heterodimer'. It may be that only the spacing of the half-sites is important; alternatively the difference between their sequences may also be important. The critical aspect is that the $a2$ half-site in the $a1/a2$ element cannot be recognized productively by the $a2$ because the other half of the element does not allow cooperative binding by both halves of the $a2$ dimer. Hence, the $a1/a2$ element is recognized productively only if both $a1$ and $a2$ are present in the same cell. In such a system, the specificity of an operator depends on three major variables: (1) the presence of short DNA sequences to which particular factors bind specifically; (2) the presence of two or more such sequences in close proximity such that cooperative binding may occur; (3) cooperative binding may be dependent critically on the spacing between the sequences. Only when the correct combination of regulatory proteins is present in a cell is the operator affected.

Discussion

We have identified, by DNA sequence comparisons, two separate sequence motifs that seem to confer two different forms of cell-type-specific repression ($a1/a2$ repression in diploids and $a2$ repression in α cells). To demonstrate that these sequences do confer cell-type-specific repression, rather than some other feature that these genes may have in common, we have excised small fragments containing each of these 'elements' and inserted them into the promoter of an unrelated gene, *CYC1*. In these constructions the *CYC1* promoter comes under the appropriate cell-type control, demonstrating that a promoter can be brought under a particular type of regulation merely by the insertion of the appropriate element into it (Figs 3, 4). Therefore, we believe that the sequence (T/C)C(A/G)TGTNN(A/T)NANN-TACATCA (Table 1) is sufficient to confer $a1/a2$ -mediated repression and that the sequence GCATGTAATACCCAAA-AAGGAAATTTACATGG (or a similar sequence) is sufficient to confer $a2$ -mediated repression. This conclusion is consistent with other results (A. Johnson and I. Herskowitz, personal communication) showing that an $\alpha2$ - β -galactosidase fusion protein binds specifically to the latter sequence *in vitro*.

These two sequences define two 'eukaryotic operator' sequences, in that they are short DNA sequences that repress adjoining genes when present in *cis*. The $a1/a2$ element seems to function to full effect only when placed between the UAS (an upstream sequence required for efficient transcription²⁸) and the TATA box, but not necessarily next to either promoter element. Hence,

it is unlikely to function like a prokaryotic operator, which directly excludes RNA polymerase from binding to the promoter²⁹. It could act by blocking the transmission of some signal from the UAS to the TATA box, as suggested by the effect of the *lexA* operator on the *GAL1* promoter³⁰.

How does the $a1/a2$ element differ from the $a2$ element such that the former is not recognized by $a2$ -mediated repression? The $a1/a2$ and $a2$ elements have related, but distinct sequences; both are inverted repeats and both carry the same sequence motif (ATGT...ACAT) in inverted orientation. The first and most marked difference between the two operators is the spacing between the inverted repeats; the common ATGT...ACAT motif is separated by 11 bp in the $a1/a2$ element, but by ~24 bp in the $a2$ element. Second, the sequence motifs found in inverted orientation may not be identical (see Fig. 5a).

What does the presence of the $a1$ gene product in the cell do to extend the action of $a2$ to include the $a1/a2$ element? Protein sequence data suggest that $a1$ is a DNA binding protein; the carboxy-terminal domains of $a1$ and $a2$ are homologous¹¹, and we know that the $a2$ protein is a sequence-specific DNA binding protein (A. Johnson, personal communication). Furthermore, the homologous region includes the helix-turn-helix motif found in prokaryotic DNA binding proteins¹⁷. We therefore prefer the model in which $a1/a2$ exists on the DNA as a heterodimer, with each protein binding to one half of the $a1/a2$ element (to one 'half-site'; Fig. 5b).

The model for the specificity of an operator described in Fig. 5b involves the combinatorial interaction of regulatory proteins on the DNA; in such a system different proteins that recognize the same DNA sequence (the same half-site) could perform very different functions because of the different protein-protein interactions they can make. Thus, it is significant that the *MATa1* and *MATa2* proteins show homology to the homoeo domain¹¹, which also seems to conserve the prokaryotic helix-turn-helix motif and is therefore believed to be a DNA binding domain³¹. The genes from *Drosophila* that contain the domain¹²⁻¹⁴ have quite distinct functions involved in different aspects of the determination of cell fate. It seems probable that the different functions of these different proteins are encoded by their non-homologous regions. These regions could either specify a second DNA-binding specificity, or, analogously to $a1$ and $a2$, they could specify a protein-protein interaction.

We find the idea that homoeo proteins may interact in a combinatorial manner with each other and with other proteins attractive, because (as with $a1$ and $a2$) different combinations of these selector genes in different cells would activate different cell-type-specific genes. In this way the combinatorial genetic addresses^{32,33} of different cells could be 'read' directly by the promoters of the different cell-type-specific genes.

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1. Mortimer, R. K. & Hawthorne, D. C. *Yeasts* 1, 385 (1969).
2. Mackay, V. L. & Manney, T. R. *Genetics* 76, 255-271 (1974).
3. Mackay, V. L. & Manney, T. R. *Genetics* 76, 273-288 (1974).
4. Nasmyth, K. A., Tatchell, K., Hall, B., Astell, C. R. & Smith, M. *Nature* 289, 244-250 (1981).
5. Tatchell, K. *et al.* *Cell* 27, 25-35 (1981).
6. Strathern, J. N., Hicks, J. B. & Herskowitz, I. *J. molec. Biol.* 147, 357-372 (1981).
7. Klar, A. J. S., Strathern, J. N. & Hicks, J. B. in *Microbial Development* (eds Losick, R. & Shapiro, L.) 151-195 (Cold Spring Harbor Laboratory, New York, 1984).
8. Wilson, K. & Herskowitz, I. *Molec. cell. Biol.* 4, 2420-2427 (1984).
9. Klar, A. J. S., Strathern, J. N., Broach, J. B. & Hicks, J. B. *Nature* 289, 239-244 (1981).
10. Jensen, R., Sprague, G. F. & Herskowitz, I. *Proc. natn. Acad. Sci. U.S.A.* 80, 3055-3059 (1983).
11. Shepherd, J. C. W., McGinnis, W., Carrasco, A. E., de Robertis, E. M. & Gehring, W. J. *Nature* 310, 70-71 (1984).
12. McGinnis, W., Levine, M. S., Hafen, E., Kuroiwa, A. & Gehring, W. J. *Nature* 308, 428-433 (1984).
13. Scott, M. P. & Weiner, A. J. *Proc. natn. Acad. Sci. U.S.A.* 81, 4115-4119 (1984).
14. Poole, S. J., Kauvar, L. M., Drees, B. & Kornberg, T. *Cell* 40, 37-43 (1985).
15. Carrasco, A. E., McGinnis, W., Gehring, W. J. & de Robertis, E. M. *Cell* 37, 409-414 (1984).
16. McGinnis, W., Garber, R. L., Wirz, J., Kuroiwa, A. & Gehring, W. J. *Cell* 37, 403-408 (1984).
17. Sauer, R. T., Yocum, R. R., Doolittle, R. F., Lewis, M. & Pabo, C. O. *Nature* 298, 447-451 (1982).

18. Kostriken, R., Strathern, J. N., Klar, A. J. S., Hicks, J. B. & Heffron, F. *Cell* **35**, 167-174 (1983).
19. Jensen, R. thesis, Univ. Oregon (1983).
20. Nasmyth, K. A. *Cell* (submitted).
21. Miller, A. M., Sternglanz, R. & Nasmyth, K. A. *Cold Spring Harb. Symp. quant. Biol.* **49**, 105-113 (1984).
22. Siliciano, P. & Tatchell, K. *Cell* **37**, 969-978 (1984).
23. Nasmyth, K. A. *Cell* (submitted).
24. Guarente, L. & Ptashne, M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2199-2203 (1981).
25. Guarente, L., Lalonde, B., Gifford, P. & Alani, E. *Cell* **36**, 503-511 (1984).
26. Staden, R. *Nucleic Acids Res.* **12**, 505-519 (1984).
27. Singh, A. *et al. Nucleic Acids Res.* **11**, 4049-4063 (1983).
28. Guarente, L. *Cell* **79**, 799-800 (1984).
29. Pribnow, D. *Biol. regul. Dev.* **1**, 219-277 (1979).
30. Brent, R. & Ptashne, M. *Nature* **312**, 612-615 (1984).

31. Laughon, A. & Scott, M. P. *Nature* **310**, 25-30 (1984).
32. Struhl, G. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7380-7384 (1982).
33. Lawrence, P. A. & Morata, G. *Cell* **35**, 595-601 (1983).
34. Astell, C. R. *et al. Cell* **27**, 15-23 (1981).
35. Staden, R. *Nucleic Acids Res.* **10**, 2951-2961 (1982).
36. Guarente, L. & Mason, T. *Cell* **32**, 1279-1286 (1983).
37. Messing, J. & Vieira, J. *Gene* **19**, 269-276 (1982).
38. Maniatis, T., Fritsch, E. F. & Sambrook, J. in *Molecular Cloning: A Laboratory Manual*, 315 (Cold Spring Harbor Laboratory, New York, 1982).
39. Miller, J. H. in *Experiments in Molecular Genetics*, 352-355 (Cold Spring Harbor Laboratory, New York, 1972).
40. Nasmyth, K. A. *Nature* **302**, 670-676 (1983).
41. Rothstein, R. *Meth. Enzym.* **101**, 202 (1983).
42. Bankier, A. T. & Barrell, B. G. in *Techniques in Life Sciences* Vol. B508, 1-34 (Elsevier, Amsterdam, 1983).

LETTERS TO NATURE

Combined MERLIN/VLA observations of the superluminal quasar 3C179

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MERLIN¹ (the Multi-Element Radio-Linked Interferometer Network) at Jodrell Bank and the VLA² (the Very Large Array) in New Mexico are complementary instruments, representing the latest generation of aperture-synthesis radio telescopes; the VLA comprises 27 antennas, which, in the A-configuration, provide spacings of 0.6–36 km, whereas MERLIN is an array of six antennas with spacings of 6–134 km. Thus, MERLIN provides a factor of four greater resolution than VLA, whereas the shorter spacings of the latter provide essential information on large-scale structure. We present here the first map to be produced by combining data from MERLIN and the VLA. The new map, of the quasar 3C179 (redshift, $z = 0.846$), is a considerable improvement on maps made from the individual arrays and reveals greater detail, particularly in the quasar's jet, which seems to be splitting or disrupting.

3C179 is unusual in that it exhibits superluminal motion in its core³ yet its extended structure is similar to that of many other classical double or triple sources, whose ejection axes are thought to lie close to the plane of the sky. To study the large-scale structure in detail, 3C179 was observed at wavelength (λ) 18 cm for 24 h using MERLIN, giving a resolution of ~ 0.25 arc s. The instrument's sensitivity to extended emission on a scale ≥ 3 arc s is limited by the shortest baseline. The VLA was used in the A-configuration to obtain a 20-min extended 'snapshot', giving a reliable representation of structure smaller than ~ 20 arc s, with a resolution of ~ 1.2 arc s.

The individual data sets were first calibrated and mapped separately to obtain the maps shown in Fig. 1a, b, which demonstrate clearly the different capabilities of the two instruments. Because MERLIN maps are produced using closure phases⁴ rather than absolute phase, absolute positional information is lost. The MERLIN data were therefore self-calibrated using clean components from the VLA map, effectively shifting the position of the MERLIN map to give the correct phase on MERLIN baselines relative to the VLA. The visibility data from the two instruments were concatenated to give baselines ranging from the minimum VLA spacing of 0.6 to 134 km, the maximum MERLIN spacing. The resultant map is shown in Fig. 1c, demonstrating detail not seen in either of the individual maps; this is to be expected because the mapping procedure is a nonlinear process.

The slight discrepancy between the values of peak surface brightness in Fig. 1b and c is attributable to a small difference between the absolute flux scales of the MERLIN and VLA data. Also, the extended emission in the combined map seems to be

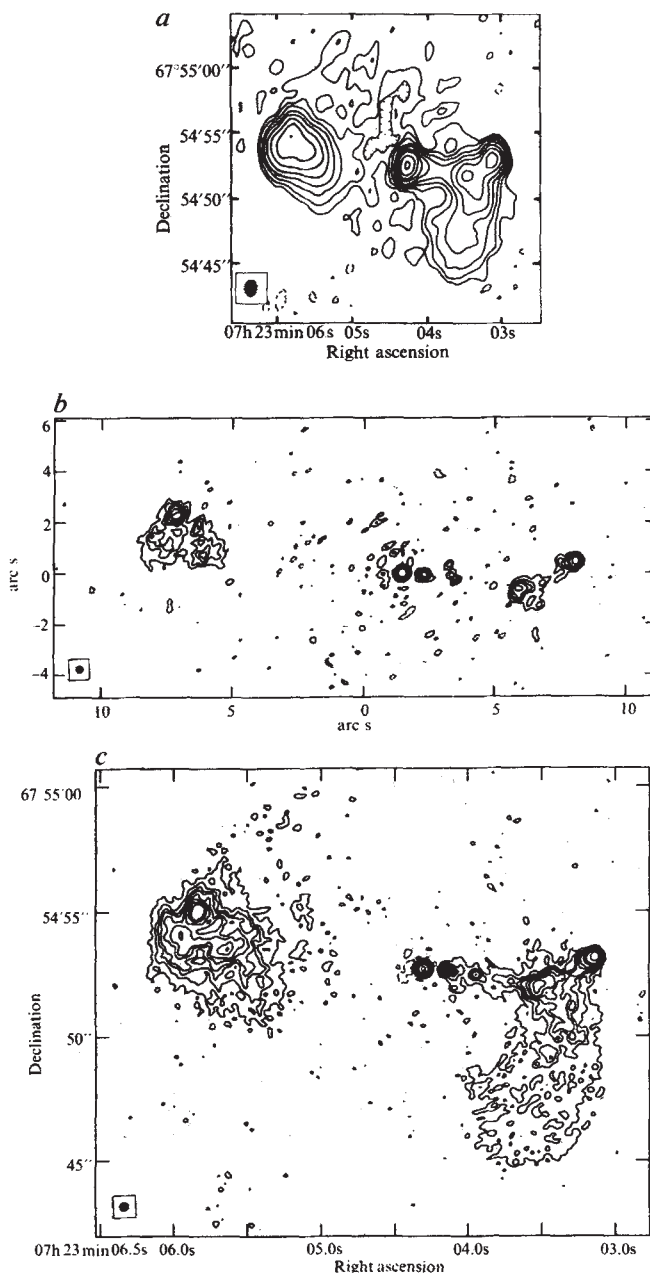


Fig. 1 Maps of 3C179 at $\lambda 18$ cm. The clean restoring beam in each case is shown in the box in the lower left corner of each map. a, VLA map. Contours are plotted at: $(-1, 1, 2, 4, 8, 16, 32, 64, 128 \text{ and } 256) \times 0.3\%$ of the peak brightness of 394 mJy per clean beam area. b, MERLIN map. Contours are plotted at: $(-1, 1, 2, 3, 4, 6, 8, 16, 32, 64) \times 0.5\%$ of the peak brightness of 374 mJy per clean beam area. c, Combined MERLIN/VLA map. Contours are plotted at: $(-1, 1, 2, 3, 4, 6, 8, 12, 16, 32, 64, 128, 256) \times 0.3\%$ of the peak brightness of 364 mJy per clean beam area.

weaker than in the VLA map. This is simply a resolution effect; when the combined map is convolved to the resolution of the VLA map, the two give identical results.

Some of the extra detail seen by virtue of the improvement in resolution over that of the $\lambda 18$ -cm VLA map is also apparent in a $\lambda 6$ -cm VLA map of Owen and Puschell⁵, which has a resolution of ~ 0.4 arc s compared with 0.28 arc s for our combined map. The extended structure agrees well with that seen in a $\lambda 73$ -cm MERLIN map⁶, which has a resolution of 1 arc s. Thus, we believe that the technique of combining data from the two arrays is reliable.

The new map of 3C179 reveals structural details that are not apparent in the maps from the individual data sets or previous maps. The jet is now clearly seen, and, like many other jets, seems to originate from a bright knot close to the core, which may be a reconfinement shock⁷. The VLBI jet⁸ emerges from the nucleus in position angle (PA) -88° ; in Fig. 1c the arc s jet is seen to bend away from this angle, reaching PA -99° approximately 3 arc s from the core. A further point of interest is the apparent splitting of the jet into two unequal flows with a possible third towards the north. Ray⁹ has suggested that Kelvin-Helmholtz instability modes of the order of $n \geq 3$ will produce a rosette-like cross-section through a jet; if these grow unhindered, they may cause a single jet to split into n jets. Alternatively, the jet may be interacting with local matter at the site of the split, causing deflection and partial disruption. A multi-frequency polarization study is planned to check for

depolarization, which may indicate the presence of thermal matter.

The overall structure of the quasar is rather asymmetrical; the relaxed structure of the eastern lobe may indicate that it is either not presently supplied by a jet, or supplied by weak and perhaps poorly collimated ejection from the core. Alternatively, the absence of a counter-jet might be explained by relativistic beaming effects. This would seem unlikely if the ejection axis lies close to the plane of the sky; however, the superluminal motion constrains the ejection axis, at least in the core, to be less than $\sim 29^\circ$ from the line of sight⁶. In common with other sources exhibiting a one-sided arc s jet, the VLBI jet is on the same side of the nucleus, implying a common cause for the asymmetries of the lobes and jets.

Our map illustrates the value of combining MERLIN and VLA data; the VLA resolution can be improved by a factor of four without the need to use a higher frequency, where steep-spectrum emission will be weaker. By using VLA $\lambda 6$ -cm maps and combined MERLIN/VLA maps at $\lambda 18$ cm, it may be possible to determine spectral indices and depolarization for source components with greater spatial resolution than hitherto.

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1. Davies, J. G., Anderson, B. & Morison, I. *Nature* **288**, 64-66 (1980).
2. Thompson, A. R., Clarke, B. G., Wade, C. M. & Napier, P. J. *Astrophys. J. Suppl.* **44**, 151-167 (1980).
3. Porcas, R. W. *Nature* **294**, 47-49 (1981).

4. Cornwell, T. J. & Wilkinson, P. N. *Mon. Not. R. astr. Soc.* **196**, 1067 (1981).
5. Owen, F. N. & Puschell, J. J. *Astr. J.* **89**, 932-957 (1984).
6. Browne, I. W. A. *et al. Nature* **299**, 788-793 (1982).
7. Sanders, R. H. *Astrophys. J.* **266**, 73-81 (1983).
8. Porcas, R. W. *Proc. IAU Symp.* **110**, 157-161 (1982).
9. Ray, T. P. *Mon. Not. R. astr. Soc.* **196**, 195-207 (1981).

Periodic minimal surfaces

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A minimal surface is one for which, like a soap film with the same pressure on each side, the mean curvature is zero and, thus, is one where the two principal curvatures are equal and opposite at every point. For every closed circuit in the surface, the area is a minimum. Schwarz¹ and Neovius² showed that elements of such surfaces could be put together to give surfaces periodic in three dimensions. These periodic minimal surfaces are geometrical invariants, as are the regular polyhedra, but the former are curved. Minimal surfaces are appropriate for the description of various structures where internal surfaces are prominent and seek to adopt a minimum area or a zero mean curvature subject to their topology; thus they merit more complete numerical characterization. There seem to be at least 18 such surfaces³, with various symmetries and topologies, related to the crystallographic space groups. Recently, glyceryl mono-oleate (GMO) was shown by Longley and McIntosh⁴ to take the shape of the F-surface. The structure postulated is shown here to be in good agreement with an analysis of the fundamental geometry of periodic minimal surfaces.

The F-surface (Schwarz's designation) discussed below would separate the disjunct tetrahedral networks in the cuprite structure (space group Pn3m). The P-surface would separate the two primitive lattices on which the Cs⁺ and Cl⁻ ions in CsCl (space group Pm3m) lie and would be similar to the silicate carcass in rho-zeolite. This P-surface would also closely resemble the surface of zero electrostatic potential in the CsCl structure. The G-surface would separate the two networks postulated by Luzzati and Spegt⁵ for strontium myristate (space group Ia3d) and structural correspondence for others of the surfaces can be found.

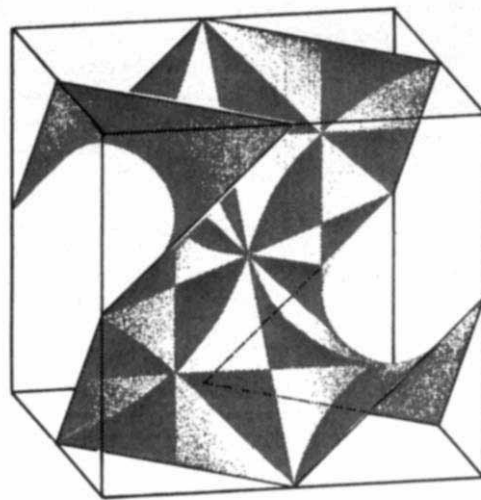


Fig. 1 The cubic unit cell of the F-surface with zero mean curvature¹. The asymmetric units (48 per cell) are shaded. The space group is Pn3m. In the cuprite Cu₂O structure, or in the double diamond structure of ice VII, this surface would separate the two non-connected networks. In the GMO structure of Longley and McIntosh⁴ the surface would be a lipid bilayer with water in the remaining space.

Several of the 18 periodic minimal surfaces described by Schoen³ (five of which were known to Schwarz) occur as structural elements. Because of their occurrence in lyotropic colloids^{4,6}, silicates⁷⁻⁹ and larger-scale biological mineralization^{10,11}, I have calculated the profiles of several of these surfaces as fundamental structural features.

If the equation for the surface is expressed (in the Monge form) as $z = f(x, y)$, the two-parameter form, then the condition

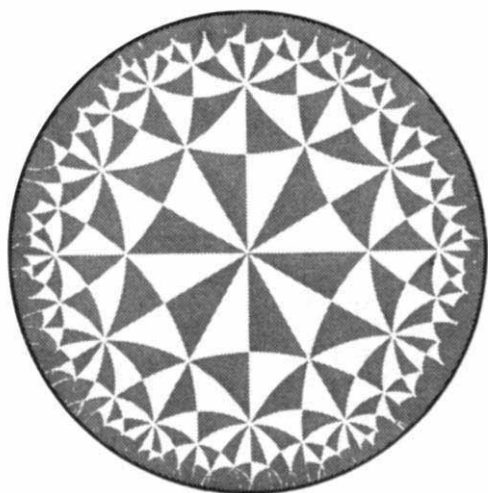


Fig. 2 A stereographic projection of the packing of the same equal triangles as in Fig. 1, but here in the hyperbolic plane, which is a space of constant negative gaussian curvature¹³. The gaussian curvature of the F-surface (Fig. 1) is not constant, but is everywhere negative (except at the umbilical points on the three-fold axes where it is zero). A surface of negative gaussian curvature cannot be mapped onto a plane without tearing or folding, but it can be represented in this way.

for zero mean curvature H , is $H = -\text{div } n = 0$ (ref. 12), which can be expanded to

$$H = \frac{(1 + f_x f_y) f_{xx} - 2 f_x f_y f_{xy} + (1 + f_x f_x) f_{yy}}{2(1 + f_x f_x + f_y f_y)^{3/2}} \quad (1)$$

where, for example, f_x represents the first partial differential of z with respect to x . This is the Laplace-Young equation.

The gaussian curvature K , the product of the principal curvatures, is given at each point by $K = (f_{xx} f_{yy} - f_{xy}^2) / (1 + f_x^2 + f_y^2)^2$. The corresponding expression for the area of the element $dx dy$ is $dA = (1 + f_x f_x + f_y f_y)^{1/2} dx dy$. An iterative method has been used, calculating the first and second derivatives of eight points nearest on a grid to the given point and adjusting the z -coordinate of the latter to make $H = 0$. K at each point has also been calculated and, as a check, the integrated gaussian curvature, which gives, from $\int K dS$, the spherical deficit of the asymmetric unit of area that is bounded by three geodesics. This quantity is also available from the three angles between the geodesics and permits the calculation of the dislocations or disclinations necessary to allow the mapping of a plane net on to the curved surface.

This calculation of z has been performed for the surfaces P, F, CLP, C(P) (Neovius surface) (in Schoen's notation). The calculated areas agree with those found using an analytical method by Schoen (who found 2.3451 for the P-surface and 3.51048 for the C(P) (Neovius surface)), except for the F-surface, where I find 1.9193 whereas Schoen has 2.4177 for the area of surface per unit cube. It is possible that there is here some difference in definition. If the asymmetric unit is taken as a triangle of sides $1/2$, $\sqrt{3}/2$, $\sqrt{2}/2$, then the area per unit cube is $3\sqrt{2}/2 = 2.1213$ and the area of these flat panels can clearly be reduced by curving them. If a hyperbolic paraboloid is fitted into a regular tetrahedron, then an area of 1.9212, only slightly greater than our value, is obtained. Schwarz's original diagram of the F-surface (called D by Schoen) is reproduced in modified form in Fig. 1. It can be visualized as the surface separating the two interpenetrating tetrahedral lattices (each of them the diamond network) in the cuprous oxide structure.

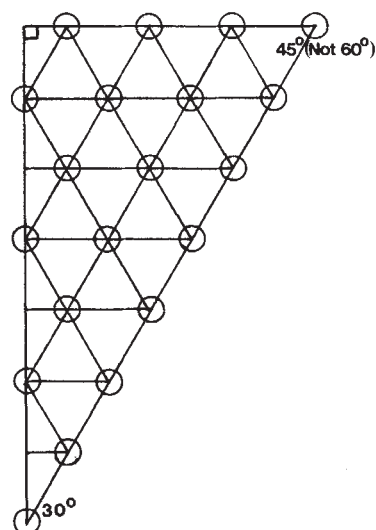


Fig. 3 A possible packing for $12 \frac{5}{24}$ molecules of GMO on one side of one of the asymmetric units of the F-surface (Fig. 1). This is an elaboration of the Longley-McIntosh structure and is based on a calculation of the area of the surface. Each molecule would have six neighbours, except that at the 45° vertex (top right), lying on the saddle point where eight triangles meet, which would have eight neighbours. The bilayer (which if planar would be hexagonally close-packed) can be tailored to the F-surface if a disclination occurs where a ring of eight instead of six molecules forms around the saddle point at which the maximum negative gaussian curvature is located.

From the gaussian curvature we can calculate the mean coordination number (CN) of molecules packed on the curved surface element. The mean CN for a plane surface must be six, but, if the surface is curved synclastically, this can be reduced. For example, if the spherical excess of an equilateral triangle with angles of 72° is 36° , then five of these triangles may meet at a point and we obtain a closed surface of icosahedral symmetry. Similarly, if the surface had a negative curvature so that the spherical deficit of a triangle were 25.71° , then seven triangles, with angles $360/7 = 51.43^\circ$, could meet at a point thus tiling an imaginary sphere (a sphere of radius i) of anticlastic curvature.

The spherical deficit for the triangle (with angles 90° , 45° and 30°), which is the unit of pattern for the F-surface, is 15° . The maximum gaussian curvature occurs at the saddle point and gives radii of curvature there of $+0.29$ and -0.29 (for a unit cube). Figure 2¹³ shows a stereographical projection of a packing of these triangles with the same local connectivity as in the F-surface, but in the hyperbolic plane with a constant gaussian curvature. Bonnet's theorem, "that with every surface of constant mean curvature $(2A)^{-1}$ there is a parallel surface of constant gaussian curvature at a distance A from it", is relevant here, but the two surfaces are infinitely far apart (because the minimal surface has a zero mean curvature). The F-surface maps onto the hyperbolic plane in multiple layers joined at the umbilical points.

The best example of a molecular structure taking the form of the F-surface is that of GMO with water, studied by Longley and McIntosh⁴. Their structure is cubic, of space group $Pn3m$, with $a = 105 \text{ \AA}$. An F-surface of this dimension would have an area of $21,160 \text{ \AA}^2$ per unit cell. Assuming, as these authors suggest, a 60% GMO content by weight, there should be (with a relative molecular mass 372.55 and a density for neat GMO of 1.028) some 1,140 molecules of GMO per cell. If they form a bilayer, as seems probable, then the area per molecule should be $37 \text{ \AA}^2$, which agrees with the value of $37.9 \pm 0.2 \text{ \AA}^2$ found by White¹⁴ for planar bilayers of GMO in the liquid-crystalline state.

There are 48 unit triangles (repeated by reflection to give eight to each saddle contained in a $1/2 \times 1/2 \times 1/2$ tetrahedron) of surface per unit cell (each with a spherical deficit of 15°) (Fig. 1). Each should carry ~ 11.8 molecules on each face to give a bilayer structure.

A disclination of $+60^\circ$ in a hexagonal network corresponds to one 5-fold instead of a 6-fold node. Thus, 12 such units are necessary to make up the 720° total if a sphere is to be covered. A disclination of -60° corresponds to the introduction of an extra segment giving a 7-fold node and a total disclination of 120° would correspond to one 8-fold node or two 7-fold nodes. In the F-surface structure, with a deficit of 15° per asymmetric unit, there is a total disclination strength of 120° for the eight units associated with each saddle point, which is where the maximum curvature is located. Thus, in the Longley-McIntosh GMO structure there should be about one dislocation, in a quasi-planar arrangement otherwise hexagonally close-packed, with an 8-fold instead of a 6-fold node associated with each of the six saddle points per unit cell. Because there are about 11.8 molecules in the asymmetric unit, this might correspond to the idealized packing shown in Fig. 3, where there would be 12 $5/24$ molecules per asymmetric unit.

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- Schwarz, H. A. *Gesammelte Mathematische Abhandlungen* Vols 1, 2 (Springer, Berlin, 1890).
- Hilbert, D. & Cohn-Vossen, S. *Geometry and the Imagination*, 271 (Chelsea, New York, 1952).
- Schoen, A. H. *NASA Tech. Note D-5541* (1970); *Not. Am. Math. Soc.* **16**, 519 (1969); **15**, 929 (1968); **15**, 727 (1968); **14**, 661 (1967).
- Longley, W. & McIntosh, T. J. *Nature* **303**, 612-614 (1983).
- Luzzati, V. & Spetz, P. A. *Nature* **215**, 701-704 (1967).

The crystallographic structure factors $F(h, k, l)$ for several of the surfaces have also been calculated, because they should also be useful geometrical invariants. For the F-surface the first 10 values are (normalized to $F(0, 0, 0) = 1.9193$ (the total area per cell) and including no atomic scattering curves: $F(0, 0, 0)$, 1.9193; $F(1, 1, 0)$, 0.4775; $F(1, 1, 1)$, 0.4866; $F(2, 0, 0)$, -0.2564; $F(2, 1, 1)$, 0.2129; $F(2, 2, 0)$, 0.2578; $F(2, 2, 1)$, 0.2789; $F(3, 1, 0)$, -0.1877; $F(3, 1, 1)$, -0.0946; $F(2, 2, 2)$, 0.2960; $F(3, 2, 1)$, 0.0952; $F(4, 0, 0)$, 0.1458.

We would expect to encounter the physical occurrence of other of the periodic minimal surfaces, particularly in liquid-crystal systems, although the F-surface discussed here has probably the smallest maximum curvature. We consider it a useful objective to calculate the profiles, curvatures and structure factors of all these periodic minimal surfaces, because they are geometrical invariants to which a number of physical structures can often be usefully referred.

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- Fontell, K. *Molec. Crystal Liq. Crystal* **63**, 59-82 (1981).
- Mackay, A. L. *Silicate Membranes as Minimal Surfaces* (Poster/Abstract 73-P1-5a Abstr. IUC Reg. Conf. Copenhagen, 1979).
- Andersson, S. *Angew. Chemie* **22**, 69-81 (1983).
- Andersson, S., Hyde, S. T. & Schneringer, H. G. von, *Zeit. f. Krist.* **168**, 1-17 (1984).
- Donnay, G. & Pawson, D. L. *Science* **166**, 1147-1150 (1969).
- Nissen, H.-L. *Science* **166**, 1150-1152 (1969).
- Weatherburn, C. E. *Differential Geometry* Vols 1, 2 (Cambridge University Press, 1939).
- Coxeter, H. S. M. *Introduction to Geometry*, 285 (Fig. 15.8c) (Wiley, New York, 1961).
- White, S. H. *Biophys. J.* **23**, 337-347 (1978).

Residence-time distributions in regions of steady-flow systems

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Questions often arise in the applied sciences concerning the transport of material through a system¹⁻⁴ (for example, a chemical plant, the cardiovascular system or a river basin). Conserved particles such as molecules, blood corpuscles or colloidal particles passing through the system spend different times in the system even when the flow is steady. The theory of residence times^{1,2} may be used to relate tracer studies to theoretical or empirical models. Existing theory mainly concerns complete systems, but a few results have been established^{2,5-7} concerning the times that particles spend in a specific region (for example, a particular phase in a chemical reactor, an organ or a backwater) while passing through the system. I show here that the distribution of these 'region residence times' may be calculated from a system model by using a formalism in which the volumes of all the other regions are reduced to zero, allowing the region distribution to be calculated by well-established methods^{1,2} that apply to systems.

Most existing theory is confined to systems with closed inlets and outlets. A closed inlet or outlet is one where the particles move solely by bulk flow: once particles enter the system they cannot re-emerge, even temporarily, at the inlet, nor, once they have left the outlet, can they re-enter the system. This restriction is needed to avoid ambiguity when a tally is taken of the particles. The residence-time density function $f(t)$ is defined by saying that $f(t)dt$ is the proportion of particles entering (or leaving) the system that will spend (or has spent) a length of time between t and $t+dt$ within the system when it is in the steady state.

To see the importance of the distribution of times that particles spend in a region of a system, consider, for example, the times

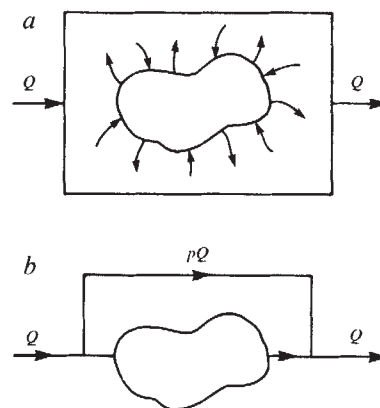


Fig. 1 a, A system with a well-mixed region; b, the system obtained by using the formalism gives the region residence-time distribution.

that molecules of a drug administered orally spend in a particular organ before being eliminated from the body, or the times that pollutant molecules discharged into a lake spend in a certain zone of the lake before eventually being carried downstream. In these examples there is no need for the region to be closed. A particle may enter a region several times during its journey through the system, or it may bypass the region altogether. Even if the region does have closed boundaries it may be possible for particles to recycle from the region outlet to the region inlet. The residence time in a region is the total time a particle spends in the region between first entering the system and finally leaving it. The residence-time density function (loosely 'distribution') $f_R(t)$ for a region is defined in the same way as the system distribution $f(t)$. Gibilaro⁵ has shown that the residence-time distribution for a well-mixed region of a system (Fig. 1a) is the sum of an exponential distribution and a spike with zero residence time that represents particles which do not enter the region during their passage through the system. Nauman⁶ has shown that the residence-time distribution for an open segment of a

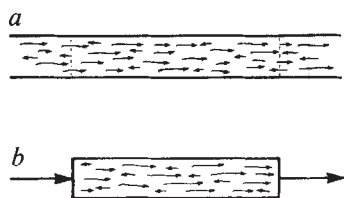


Fig. 2 Axially dispersed piston flow: *a*, showing a segment with open boundaries; *b*, showing the upstream and downstream volumes reduced to zero to find the system that gives the region residence-time distribution.

duct carrying an axially dispersed fluid (Fig. 2*a*) is exactly the same as it would be if the segment were closed. The mean residence time in a region is the ratio of the hold-up in the region to the rate of transmission through the system⁷.

Nauman's result was the solution to what had been a perplexing problem. His method was to imagine a particle undergoing a random walk and a clock that stops whenever the particle leaves the segment on which attention is focused and restarts when it re-enters. Residence time is the time the clock records between initial entry and final departure. This provides the clue to deducing the residence-time distribution for an open region. Instead of timing particles with clocks, imagine that each particle is provided with a watch which runs only when the particle is within the region. When a particle leaves the region for the last time, its watch shows the residence time. If, when outside the region, the particles could be induced to travel at infinite speed but along their proper paths, their watches could run continuously. Thus we arrive at the following formalism for deducing the region residence-time distribution from a system model. Reduce the volumes of all regions of the model other than the one of interest to zero and find the residence-time distribution of the resulting model. We can thus establish Gibilaro's and Nauman's results in an extremely simple and straightforward way.

As the portion of the system in Fig. 1*a* outside the well-mixed region is shrunk, particles entering the system reach the well-mixed zone more quickly, return to it more quickly, and so on. At the limit, returning particles return instantaneously. Removing particles from a well-mixed region and instantaneously returning them, to whatever location, has no observable physical effect. Consequently the region residence-time distribution is the same as the system distribution for the system in Fig. 1*b* and is

$$f_R(t) = p\delta(0) + [(1-p)^2 Q/h_R] \exp[-(1-p)Qt/h_R] \quad (1)$$

as Gibilaro⁵ showed. The impulse, $p\delta(0)$, represents particles that bypass the region and h_R is the steady-state hold-up of particles there. For models of modest complexity it is usually possible to deduce the bypass fraction p by *ad hoc* methods, but a general procedure is available⁸ for network models. For the case of axially dispersed piston flow (Fig. 2*a*), replacing the parts of the system upstream and downstream of the region of interest by regions with no volume gives the closed system of Fig. 2*b* and confirms Nauman's finding⁶ that the residence-time distribution for the open region in Fig. 2*a* is the same as it would be if the region were closed.

The formalism can also be used to derive new results. For example, consider a region with a single closed inlet and a single closed outlet embedded within a system (Fig. 3*a*). When the system is shrunk it becomes equivalent to that shown in Fig. 3*b*: it may be possible for particles to bypass the region; other particles either leave after one passage through the region or are recycled. The region residence-time density is

$$f_R(t) = p\delta(0) + (1-p)f_{RS}(t) \quad (2)$$

where $f_{RS}(t)$ is the residence-time density for the recycle system within the broken line in Fig. 3*b*. This density is related to the density of (once-through) sojourn times in the region^{2,9}.

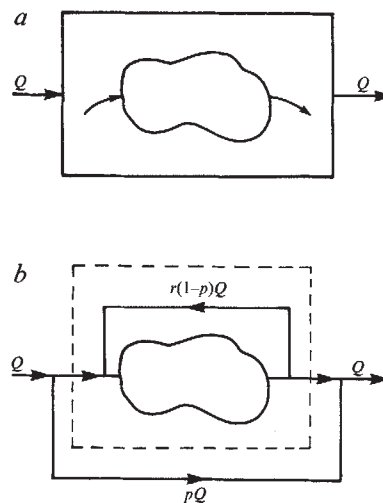


Fig. 3 *a*, A region with a closed inlet and a closed outlet embedded within a system; *b*, the system that gives the region residence-time distribution.

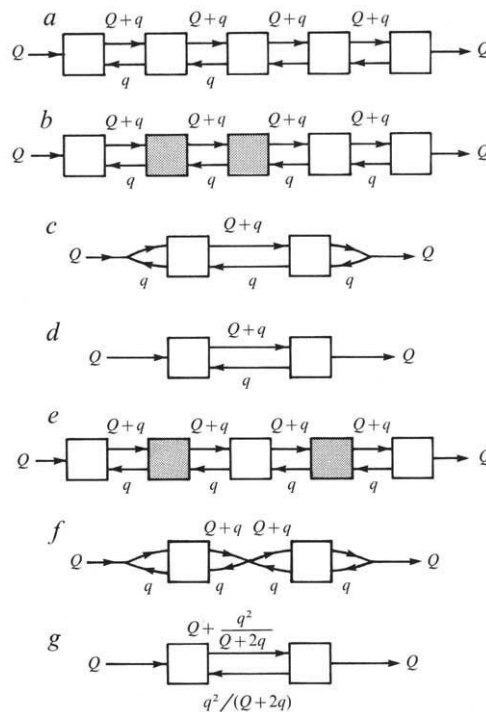


Fig. 4 Sequences of well-mixed zones with backflow: *a*, the basic model; *b*, a region consisting of two adjacent zones; *c*, the system that gives the region residence-time distribution for a region consisting of two adjacent zones according to the formalism; *d*, the system of *c*, further simplified; *e*, a region consisting of two non-adjacent zones; *f*, the system that gives the residence-time distribution for a region consisting of two non-adjacent zones; *g*, the system of *f* further simplified.

Gibilaro⁹ has provided a survey of recycle models. The bypass ratio p and recycle ratio r will be reasonably easy to establish for relatively simple models and again the general method⁸ can be used for networks.

As a further example, consider the sequence of perfectly-mixed equal-volume zones with backflow shown in Fig. 4*a*. Suppose we wish to know the distribution of residence times in a region that consists of two adjacent zones (Fig. 4*b*). By applying the formalism, the system in Fig. 4*c* is found to have the desired residence-time distribution. This can be simplified

further to give the system in Fig. 4d. Application of standard methods² shows that the residence-time density is

$$f(t) = (Q/V_R) \sqrt{1+Q/q} \{e^{-\lambda_1 Q t/V_R} - e^{-\lambda_2 Q t/V_R}\} \quad (3)$$

where $\lambda_{1,2} = 2\sqrt{1+Q/q}/(\sqrt{1+Q/q} \pm 1)$ and V_R is the region volume. Backflow cell models have been discussed by others¹⁰⁻¹²; residence-time distributions for regions consisting of several adjacent zones can be deduced from published results^{10,11} by using the formalism. We can also find the residence-time distribution for a region consisting of two non-adjacent zones (Fig. 4e). When the formalism is invoked, the zone(s) between the two zones comprising the region shrinks into a node (Fig. 4f). Of the flow from the left-hand zone to the node, a fraction $(Q+q)/(Q+2q)$ passes on to the right-hand zone and a fraction $q/(Q+2q)$ returns to the left-hand zone. As the latter is returning at once to a well-mixed zone it has just left, it can be disregarded. Thus, the significant left-to-right flow is $(Q+q)^2/(Q+2q)$, which can also be written as $Q+q^2/(Q+2q)$. As the net flow is Q , the significant right-to-left flow is $q^2/(Q+2q)$ (see Fig. 4g). It follows by comparing Fig. 4d and g that the residence-time distribution is given by equation (3), with q replaced by $q^2/(Q+2q)$.

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1. Wen, C. Y. & Fan, L. T. *Models for Flow Systems and Chemical Reactors* (Dekker, New York, 1975).
2. Nauman, E. B. & Buffham, B. A. *Mixing in Continuous Flow Systems* (Wiley, New York, 1983).
3. Shepherd, C. W. *Basic Principles of the Tracer Method* (Wiley, New York, 1962).
4. Fischer, H. B., List, E. J., Koh, R. C. Y., Imberger, J. & Brooks, N. H. *Mixing in Inland and Coastal Waters* (Academic, New York, 1979).
5. Gibilaro, L. G. *Chem. Engng Sci.* **34**, 697-702 (1979).
6. Nauman, E. B. *Chem. Engng Sci.* **36**, 957-966 (1981).
7. Buffham, B. A. *Nature* **274**, 879-880 (1978).
8. Rubinovitch, M. & Mann, U. *A.I.Ch.E. J.* **29**, 658-662 (1983).
9. Gibilaro, L. G. *Chem. Engng Sci.* **26**, 299-304 (1971).
10. Roemer, M. H. & Durbin, L. D. *Ind. Engng Chem. Fundamentals* **6**, 120-129 (1967).
11. Haddad, A. H. & Wolf, D. *Can. J. chem. Engng* **45**, 100-104 (1967).
12. Mecklenburgh, J. C. & Hartland, S. *The Theory of Backmixing* (Wiley, Chichester, 1975).

Atmospheric chlorofluoromethanes in the deep equatorial Atlantic

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Waters that leave the surface of the ocean and enter the subsurface circulation contain concentrations of CCl_3F (fluorocarbon-11) and CCl_2F_2 (fluorocarbon-12), which reflects the temporal increases of these industrially produced compounds in the atmosphere. These chlorofluoromethanes (CFMs) are extremely stable in the troposphere and in natural waters, they have no known natural sources, and their histories of release to the atmosphere are fairly well known¹. The atmospheric distributions of CCl_3F and CCl_2F_2 are not strongly dependent on latitude^{2,3}, and their surface water concentrations can be expected to come into relatively rapid solubility equilibrium with the atmosphere⁴. Recent advances in analytical techniques^{5,6} have made possible the routine use of these CFMs as oceanic tracers on a decadal timescale. The results we report here are from the first detailed surveys of CCl_3F and CCl_2F_2 distributions in the northern and tropical Atlantic Ocean. They show that CFM-bearing waters originating in the region of the Labrador Sea have reached the Equator in a well-defined western-boundary undercurrent located at a depth of about 1.6 km, in the Upper North Atlantic Deep Water. Using a simple dilution model, we calculate that this water has taken about 23 yr to reach the equatorial region, and has been diluted about five-fold by CFM-free waters.

Our measurements were carried out aboard R/V *Knorr* during Leg 7 of the Transient Tracers in the Ocean (TTO) North Atlantic Study (NAS) in September-October 1981, and during the three legs of the TTO Tropical Atlantic Study (TAS) from December 1982 to February 1983. The station numbers and locations of these expedition legs are shown in Fig. 1. Concentrations of CCl_3F and CCl_2F_2 were measured at about 15-30 depths at each station, and in samples of marine air, using shipboard electron-capture gas chromatography. The techniques for measuring CFMs have evolved considerably during the course of this work: the NAS measurements were made using a modified version of the technique used earlier in the North Pacific⁵, and the TAS measurements were made by a semi-automated low-blank version of the technique used in the Greenland and Norwegian seas⁶. Seawater samples were collected in Niskin bottles and carefully transferred to 100-cm³ glass syringes to avoid contact with shipboard air, which was generally contaminated in CFMs. Measured seawater volumes of ~30 cm³ were injected into a stripping chamber, where the dissolved CFMs were purged by a stream of purified carrier gas and were collected on a low-temperature trap for injection into the gas chromatograph using a Porasil C separating column. Impurities with long elution times were removed using standard pre-column techniques. The peaks were detected by a ⁶³Ni electron-capture detector operating in constant current mode, and amounts were determined by peak height measurement on NAS and by digital integration on TAS. Seawater samples were analysed within a few hours of collection. Measured volumes of clean ambient air (~3 cm³) drawn from a continuously pumped outdoor sampling line and dried with potassium carbonate on NAS and magnesium perchlorate on TAS were analysed by injection into the same low-temperature sampling system.

The system was calibrated by frequent analyses of compressed air secondary standards in various aliquot sizes. The values we report are given on the Scripps Institution of Oceanography (SIO) 1984 calibration scale, which is based on primary standards with near-atmospheric CCl_3F and CCl_2F_2 concentrations prepared in the laboratory by volumetric dilution. The estimated accuracy of this scale is ~1.5% for CCl_3F and ~0.3% for CCl_2F_2 . We have also carried out direct intercomparisons between our standards and those used in the Atmospheric Lifetime Experiment (ALE)⁷ which demonstrate agreement within expected combined errors: values reported on the ALE scale are 2.5% higher than the SIO 1984 scale for CCl_3F and 1.9% lower than the SIO 1984 scale for CCl_2F_2 .

The precision of the seawater measurements, as estimated from the standard deviation of replicate analyses, is about 4% or 0.02 pmol kg⁻¹, whichever is greater, for both CFMs on NAS, and about 1% or 0.005 pmol kg⁻¹, whichever is greater, for both CFMs on TAS. Systematic sampling and handling errors are estimated to be on the same order.

To assess past surface-water CFM concentrations, we have reconstructed the temporal variations in high-latitude Northern Hemisphere tropospheric CCl_3F and CCl_2F_2 mixing ratios in the same way as we have done for the Greenland and Norwegian seas⁶. For the period since 1976, we have used direct measurements^{2,3,8}, corrected to the SIO 1984 calibration scale. For 1933-76, we have integrated annual CFM release figures⁹, assuming tropospheric residence times of 50 yr for CCl_3F and 90 yr for CCl_2F_2 , and have normalized these results to match the direct measurements in 1976. These data show monotonic increases in the tropospheric concentrations of both compounds, with build-up of CCl_3F beginning somewhat later but rising more rapidly, so that there has been a steady increase in the $\text{CCl}_3\text{F}/\text{CCl}_2\text{F}_2$ ratio from zero in 1937 to about 0.57 in 1975. Following the discovery that the CFMs represent a threat to the Earth's ozone layer¹⁰, the percentage rates of increase of both of these compounds have gradually decreased to about 6% per year, and there has been no significant change in the tropospheric $\text{CCl}_3\text{F}/\text{CCl}_2\text{F}_2$ ratio since about 1976.

Each of the four TAS hydrographic sections which cross the western boundary of the tropical Atlantic (Fig. 1) show pro-

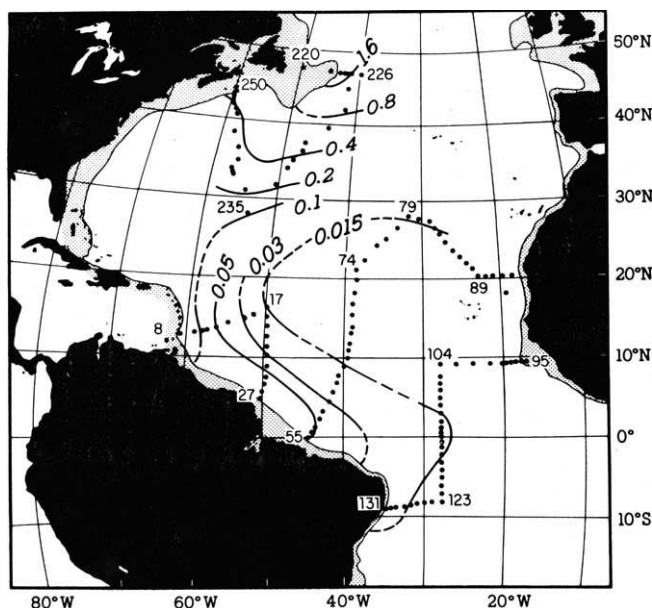


Fig. 1 Station locations and contours of CCl_3F concentrations on the $\sigma_{1.5} = 34.63 \text{ kg m}^{-3}$ isopycnal surface. Stations 220–250 in the northwestern Atlantic are from the NAS expedition and Stations 8–27 and 55–131 in the tropical Atlantic are from the TAS expedition. CCl_3F contours are in units of pmol kg^{-1} , and are spaced at approximately logarithmic intervals in which each successive contour above $0.05 \text{ pmol kg}^{-1}$ represents a doubling in concentration.

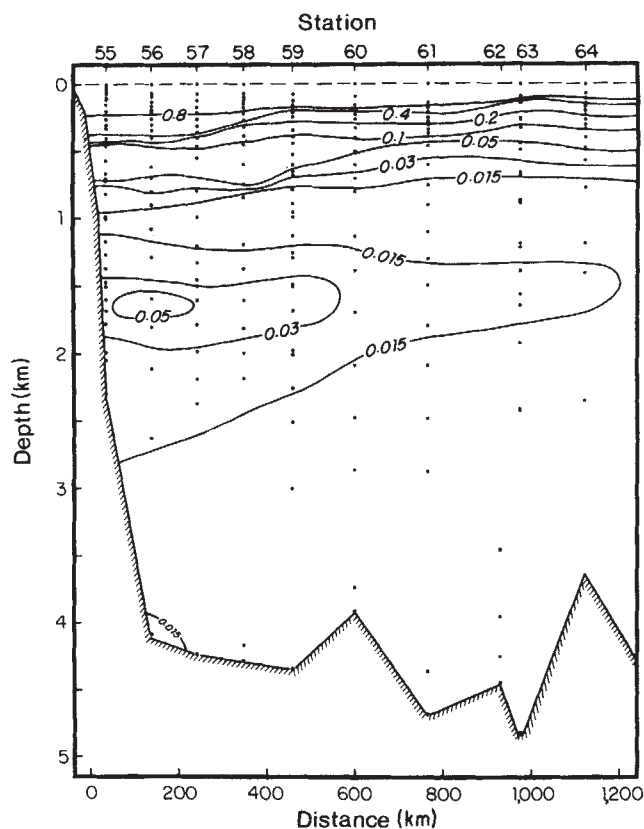


Fig. 2 Concentration of CCl_3F in pmol kg^{-1} on the TAS vertical section extending from Station 55 near the South American continent at the Equator to Station 64 near the Mid-Atlantic Ridge. The contour intervals are as in Fig. 1.

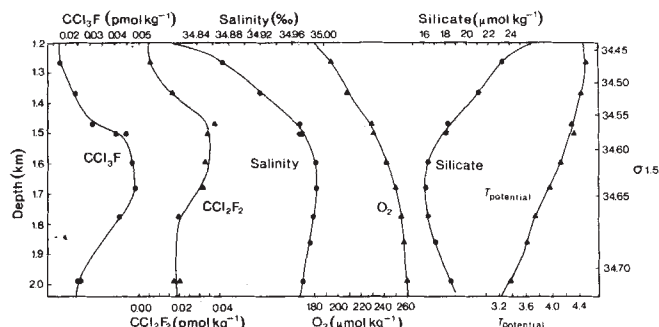


Fig. 3 Vertical profiles of CCl_3F , CCl_2F_2 , salinity, oxygen, silicate and potential temperature at TAS Station 55 ($0^\circ 03' \text{ N}$, $44^\circ 10' \text{ W}$) versus depth. Corresponding values of the potential density anomaly $\sigma_{1.5}$ in kg m^{-3} are labelled along the right ordinate.

nounced maxima in CCl_3F and CCl_2F_2 concentrations at depths of 1.5–1.7 km, with the highest concentrations lying near the South American continent and with detectable maxima extending as much as 1,200 km seaward. The CCl_3F section in Fig. 2, which extends from Station 55 near the continental margin at the Equator to Station 64 near the Mid-Atlantic Ridge, shows an example of this pattern. The CFM concentrations in this mid-depth feature also decrease toward the south, with maximum CCl_3F values ranging from $\sim 0.1 \text{ pmol kg}^{-1}$ in the section at 13° N to $\sim 0.015 \text{ pmol kg}^{-1}$ in the section at 9° S . The CFM data thus suggest that this feature is produced by a southward-flowing western boundary undercurrent.

The relationship of this feature to the regional hydrography is shown in Fig. 3 by the depth profiles of CCl_3F , CCl_2F_2 , salinity, O_2 , silicate and potential temperature (the temperature a parcel of water would have if moved adiabatically to the sea surface) at TAS Station 55. The maxima in CCl_3F and CCl_2F_2 coincide with a maximum in salinity and a minimum in silicate which mark the core of the Upper North Atlantic Deep Water (UNADW)¹¹. The potential density anomaly $\sigma_{1.5}$ (defined¹² by $\sigma_{1.5} = \rho_{1.5} - 10^3$, where $\rho_{1.5}$ is the density¹³ expressed in kg m^{-3} that a parcel of seawater would have if moved adiabatically to the depth where the pressure is 1,500 dbar) of this layer is $\sim 34.63 \text{ kg m}^{-3}$.

Tropical Atlantic waters in the potential density range of the UNADW are influenced by waters originating both in the Mediterranean Sea and in the region of the Labrador Sea. The salinity maximum of the UNADW is of Mediterranean origin, although in the western tropical Atlantic it has been truncated, and thus moved to a greater potential density, by the intrusion from the south of the overlying low-salinity Antarctic Intermediate Water (AAIW). Similarly, the silicate minimum, which is characteristic of waters of both Mediterranean and Labrador Sea origin, is truncated by the intrusion of overlying high-silicate AAIW. We emphasize that the coincidence of the depths of the CFM maxima with the extrema in salinity and silicate results simply from truncation of the profiles by low-CFM AAIW and does not necessarily indicate a common source region for these species in the UNADW.

The truncation of the UNADW extrema by the AAIW illustrates a major difficulty in using the core layer method to trace the waters in the UNADW to their source regions. We have instead evaluated the origin of the high CFMs in the UNADW by isopycnal layer analysis¹² using the $\sigma_{1.5} = 34.63 \text{ kg m}^{-3}$ potential density surface. This surface coincides with the UNADW CFM maxima we observe in the western tropical Atlantic and is characterized in this region by a potential temperature of $\sim 4.0^\circ \text{ C}$ and a salinity of $\sim 34.98\text{‰}$. Towards the north-east, the potential temperature and salinity on this surface increase in response to the influence of the Mediterranean outflow, reaching values of $\sim 5.1^\circ \text{ C}$ and $\sim 35.20\text{‰}$ at the northernmost TAS stations in the Canary Basin. Towards the north-west, our NAS data show decreases in potential temperature and salinity on this

isopycnal surface as the proportion of Mediterranean water is reduced. This effect is most pronounced at our northernmost stations near the Grand Banks, where this surface intersects a western boundary current of Labrador Sea Water (LSW) which is marked by decreases in potential temperature and salinity from $\sim 3.6^\circ\text{C}$ and $\sim 34.92\%$ at Station 226 to $\sim 3.3^\circ\text{C}$ and $\sim 34.86\%$ at Station 222. Throughout our study area, the depth of this isopycnal surface lies at ~ 1.6 km, except near the Grand Banks where it shoals to ~ 1.0 km.

The potential density and formation rate of LSW during 1948–75 have been evaluated¹⁴ using its potential vorticity minimum as a tracer. Because that study used an earlier equation of state, its values of $\sigma_{1.5}$ must be reduced by 0.03 kg m^{-3} to place them on the same scale as ours. Thus, our $\sigma_{1.5} = 34.63\text{ kg m}^{-3}$ isopycnal surface lies near the lower limit of the LSW potential density range, $\sim 0.03\text{ kg m}^{-3}$ less than the most common density of the LSW core. Based on temporal variations in salinity, the $\sigma_{1.5} = 34.63\text{ kg m}^{-3}$ surface in the Labrador Sea is believed¹⁴ to have been actively renewed during 1948–63 and to have been relatively isolated from renewal subsequently during 1964–71.

The geographical distribution of CCl_3F concentrations on the $\sigma_{1.5} = 34.63\text{ kg m}^{-3}$ isopycnal surface at our TAS and NAS station locations is shown by the contours in Fig. 1. As an approximate compensation for the 1-yr difference in expedition dates, we have increased the values at the NAS stations by 6% to make the contours in this region approximately synoptic with the TAS measurements. In the equatorial region, these distributions do not show increasing CFM concentrations as one moves along the isopycnal surface from the western boundary of the Atlantic towards the high salinity water which extends outward from the Mediterranean. In the western Atlantic, however, CFM concentrations on the isopycnal surface increase steadily towards the Labrador Sea source region in the north. As well as these geographical relationships, we note that increasing CFM concentrations correlate with decreasing salinity everywhere on this isopycnal surface, except in the region of TAS Stations 79–82, where the influence of CFMs from the Mediterranean is clearly reflected by a correlation between increasing CFM concentrations and increasing salinity. We therefore conclude that the CFMs in the UNADW of the tropical western Atlantic must originate in the region of the Labrador Sea.

We have used a simple model to calculate the approximate time required for water to be transported from the surface of the Labrador Sea to the UNADW in the western tropical Atlantic, as well as the degree of dilution which occurs during this process. We have assumed that CCl_3F and CCl_2F_2 concentrations in Labrador Sea surface waters during wintertime convection are in approximate solubility equilibrium with the atmosphere, and that during their southward transport in a western boundary undercurrent they are diluted by mixing with surrounding waters which are essentially CFM-free. In this model, the transport time for the CFM-bearing component is given by the $\text{CCl}_3\text{F}/\text{CCl}_2\text{F}_2$ ratio, and the degree of dilution is given by the concentrations.

In these calculations, we have used the temporal variations in tropospheric CFM mixing ratios discussed above, and solubilities in LSW of 3.5°C potential temperature and 34.9% salinity, expressed in terms of the F solubility function¹⁵, of $0.0213\text{ mol kg}^{-1}\text{ atm}^{-1}$ for CCl_3F and $0.00533\text{ mol kg}^{-1}\text{ atm}^{-1}$ for CCl_2F_2 (M.J.W. and R.F.W., unpublished gas chromatographic solubility measurements).

The NAS measurements of LSW near the Grand Banks at $\sim 47^\circ\text{N}$ and near Nova Scotia at $\sim 42^\circ\text{N}$ show $\text{CCl}_3\text{F}/\text{CCl}_2\text{F}_2$ ratios of 2.25 ± 0.1 , which correspond to an equilibrium atmospheric ratio of 0.56 ± 0.02 . This ratio is indistinguishable from the atmospheric ratio of 0.56 in 1981 during the NAS expedition, and because of the constancy of the $\text{CCl}_3\text{F}/\text{CCl}_2\text{F}_2$ ratio during the late 1970s, our ratio measurements can only constrain the mean transport time of these waters to < 9 yr. Calculated dilution factors for the LSW found in this region are also poorly constrained, by the uncertainty in the transport time and by the

range of the measured concentrations, to between zero and three-fold.

The TAS measurements of UNADW in the western boundary undercurrent of the equatorial Atlantic show $\text{CCl}_3\text{F}/\text{CCl}_2\text{F}_2$ ratios ranging from 1.2 to 1.5, with a mean value of ~ 1.4 . Although we expect from the southward decrease in CFM concentrations that there is a corresponding decrease in $\text{CCl}_3\text{F}/\text{CCl}_2\text{F}_2$ ratio between 13°N and 9°S , we are unable to detect a significant trend because of the large error associated with the ratio measurement at these low concentrations. Including analytical errors, the mean atmospheric $\text{CCl}_3\text{F}/\text{CCl}_2\text{F}_2$ ratio in equilibrium with these waters is 0.35 ± 0.11 , which corresponds to the atmospheric composition in 1960 ± 5 yr. The degree of dilution which this water has experienced is very strongly dependent on the uncertainty in the calculated transport time because the concentrations of CCl_3F and CCl_2F_2 in the atmosphere increased approximately seven-fold and four-fold, respectively, between 1955 and 1965. For surface water equilibrated with the atmosphere in 1960, we calculate that the CCl_3F concentration of 0.05 pmol kg^{-1} found near the South American continent at the Equator has been diluted approximately five-fold by CFM-free water.

Thus, our data show that waters originating in the region of the Labrador Sea have transported atmospheric CFMs into the equatorial Atlantic along a western boundary undercurrent of UNADW, a distance of about 10,000 km, in about 23 yr. This corresponds to a mean velocity of about 1.4 cm s^{-1} . In the equatorial region, the CFM distributions show that this western-boundary flow crosses into the Southern Hemisphere to at least 9°S and suggest that the flow also extends eastward along the Equator.

It is important to recognize that the transport times and dilution factors we have calculated are strongly model dependent. Because atmospheric CFM concentrations have tended to rise exponentially rather than linearly, if subsurface mixing occurs between CFM-bearing waters of different ages, the resulting apparent age will be biased in favour of the younger component. Also, the assumption of atmospheric equilibrium in surface waters may be inappropriate in convective processes and under such conditions the CFM ratios may be affected by kinetics. Further research in the Labrador Sea and in other areas of active water-mass formation is essential for quantifying these relationships.

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1. *Halocarbons: Effects on Stratospheric Ozone* (National Academy of Sciences, Washington, DC, 1976).
2. Cunnold, D. M. *et al.* *J. geophys. Res.* **88**, 8379–8400 (1983).
3. Cunnold, D. M. *et al.* *J. geophys. Res.* **88**, 8401–8414 (1983).
4. Broecker, W. S. & Peng, T.-H. *Tellus* **26**, 21–35 (1974).
5. Gammon, R. H., Cline, J. & Wisegarver, D. *J. geophys. Res.* **87**, 9441–9454 (1982).
6. Bullister, J. L. & Weiss, R. F. *Science* **221**, 265–268 (1983).
7. Rasmussen, R. A. & Lovelock, J. E. *J. geophys. Res.* **88**, 8369–8378 (1983).
8. Rasmussen, R. A. & Khalil, M. A. K. in *Proc. NATO Advanced Study Inst. Atmospheric Ozone* (ed. Aiken, A. C.) 209–231 (Federal Aviation Administration, Washington, DC, 1980).
9. *Production, Sales and Calculated Release of Chlorofluorocarbons 11 and 12 through 1982* (Chemical Manufacturers Association, Washington, DC, 1983).
10. Molina, M. J. & Rowland, F. S. *Nature* **249**, 810–812 (1974).
11. Wüst, G. *Wiss. Ergebn. dt. atlant. Exped. 'Meteor'* **6**, 109–288 (1935).
12. Lynn, R. J. & Reid, J. L. *Deep-Sea Res.* **15**, 577–598 (1968).
13. Millero, F. J., Chen, C.-T., Bradshaw, A. & Schleicher, K. *Deep-Sea Res.* **27A**, 255–264 (1980).
14. Talley, L. D. & McCartney, M. S. *J. phys. Oceanogr.* **12**, 1189–1205 (1982).
15. Weiss, R. F. & Price, B. A. *Mar. Chem.* **8**, 347–359 (1980).

Sulphate and nitrate concentrations in snow from South Greenland 1895–1978

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An understanding of the phenomenon of acid rain requires the identification of the sources of the species affecting the pH of rainwater, both natural and anthropogenic, and their temporal and spatial development. The scant data concerning the historical development of the acidity in precipitation are from urban regions or their vicinity, where local effects dominate and obscure the hemispherical pattern¹. The Greenland ice sheet allows us to trace the evolution of the acid rain in a remote location that is free from local effects. Sulphuric and nitric acids are the two species that dominate the acidity in precipitation^{2–4}. We report here measurements of $[\text{SO}_4^{2-}]$ and $[\text{NO}_3^-]$ in firn samples spanning the period 1895–1978. Samples, each covering 1 yr, were taken from a 70-m core drilled at Dye 3, South Greenland; $[\text{NO}_3^-]$ and $[\text{SO}_4^{2-}]$ both increased by a factor of ~ 2 during the period. By comparing the recent concentrations of nitrate and sulphate with those resulting from natural sources, we conclude that anthropogenic emissions of the precursors (NO_x , SO_2) had already surpassed natural sources in the late 1950s.

In 1980 a shallow electromechanically drilled core was collected near Dye 3 ($65^\circ 11' \text{N}$, $43^\circ 30' \text{W}$) in the frame of the US–Danish–Swiss Greenland Ice Sheet Program. The main purpose of this core was to provide samples for studying the influence of the 11-yr solar cycle on the ^{10}Be concentration in the firn⁵. As a supplementary study, anion concentrations were determined by ion chromatography as possible correction factors to eliminate ‘meteorological noise’ in the ^{10}Be data. To avoid contamination, the drill site was located 2 km north of the radar station and perpendicular to the main wind direction. In the field, the acidity was qualitatively determined by solid electrical conductivity measurement (ECM)⁶ and a small strip of the core was cut for continuous $\delta^{18}\text{O}$ analysis with a resolution of eight samples per calculated year. After field sampling, the cores were packed in polyethylene tubes and sent back to the laboratory. The volcanic Katmai eruption, which took place in 1912 in Alaska, is clearly visible in the ECM record and served as an internal check on the accuracy of the timescale used. The $\delta^{18}\text{O}$ and ECM results showed the mean snow accumulation rate at Dye 3 to be 50-cm water equivalent yr^{-1} and provided an age/depth relation which allowed us to cut the core into annual samples. To remove external contamination before chemical analysis, the outer layer of the firn was removed with a stainless steel microtome knife. All sample treatment was done in a cold room (-20°C). The samples were melted in a covered polyethylene beaker and aliquots were taken for chemical analysis. To check whether the sampling and melting procedure introduces any noticeable background, we treated ultra-pure water in the same manner as the samples. This water was frozen in the polyethylene beaker in the cold room and re-melted in the laboratory. Blank values were below 8 parts per 10^9 (p.p.b.) (ng per g ice) for the measured species; the reproducibility of the measurements was better than 5%. Figures 1 and 2 show $[\text{NO}_3^-]$ and $[\text{SO}_4^{2-}]$ during the period 1895–1978. To reveal the general trend, a spline-smoothed curve has been drawn through the points. Nitrate and sulphate show an increasing trend with a large year-to-year fluctuation; our measured concentrations can be compared with published data^{7–9}. For the years 1895–1904 we measured a mean sulphate level of 36 p.p.b., which is higher than for the two time periods 1539–1577 and 1773–1791, where the sulphate level was determined by Herron⁷ to be 22 p.p.b.

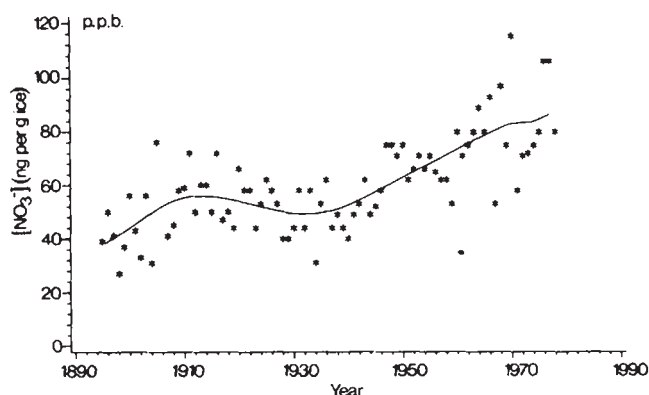


Fig. 1 Annual $[\text{NO}_3^-]$ in a shallow core from Dye 3, South Greenland. The general trend is shown with a spline-smoothed curve.

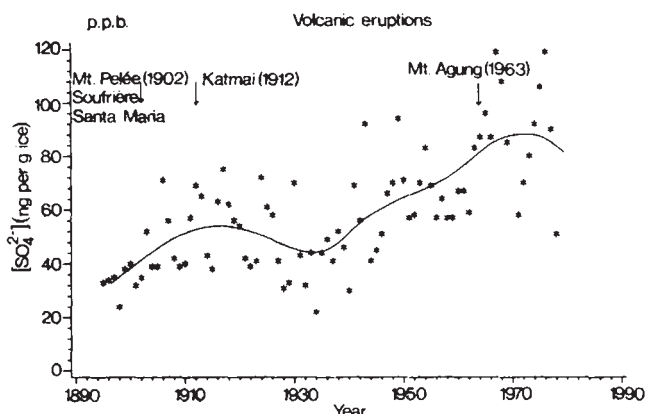


Fig. 2 Annual $[\text{SO}_4^{2-}]$ in a shallow core from Dye 3, South Greenland. The general trend is shown with a spline-smoothed curve.

Under unfavourable meteorological conditions the radar station is a possible source of contamination, especially for sulphate. However, the measured $[\text{SO}_4^{2-}]$ of 90 p.p.b. for the recent time period 1973–1978 agrees well with Herron's level of 85 p.p.b. for the time period 1975–1981, which was determined in a pit study much further from the Dye 3 station than was our drilling site. Furthermore, our sulphate and nitrate data do not show a discontinuity over the year 1961, when the radar station was constructed. We conclude, therefore, that the contamination attributable to emissions from the radar station is small. We found no indication that the occurrence of melt layers in the Dye 3 firn core influences $[\text{NO}_3^-]$ and $[\text{SO}_4^{2-}]$.

The nitrate levels measured by Herron⁷ are generally slightly higher than ours (Herron's 1884–91, 51 p.p.b., our 1895–1904, 44 p.p.b.; Herron's 1975–81, 114 p.p.b., our 1973–78, 86 p.p.b.). The high nitrate value we found for 1906 is surprising; we cannot exclude contamination of this sample, particularly as $[\text{SO}_4^{2-}]$ is also relatively high and in Rasmussen's Dye 3 data⁸, $[\text{NO}_3^-]$ for 1906 is not elevated. As a consequence of the 1908 Tunguska impact in Siberia, Turco *et al.*¹⁰ have calculated that NO_x concentrations in the lower stratosphere would be elevated by an order of magnitude for a period over 1 yr. The years 1908–10 do not, however, show a higher $[\text{NO}_3^-]$, so that the predicted enhancement of $[\text{NO}_x]$, if it occurred, did not penetrate into the troposphere. Similar conclusions were reported by Rasmussen *et al.*⁸, based on their measurements at Dye 3 and Camp Century.

The concentration depends on three factors: the scavenging process, the transport of air masses from the source to the receptor and the source strength. We ascribe the general trend in anion concentrations to increasing source strength and the short-time fluctuations to the variability in transport processes and scavenging.

Although NO_3^- and SO_4^{2-} are mainly incorporated as HNO_3

and H_2SO_4 in the ice¹¹⁻¹³, HNO_3 and H_2SO_4 are not emitted directly into the atmosphere. The precursors for HNO_3 are NO and NO_2 , and for H_2SO_4 are SO_2 , dimethyl sulphide (DMS) and H_2S . All these species are oxidized in the atmosphere, either in the gas phase or in cloud droplets¹⁴ to form the two acids, which are then effectively removed by precipitation. The global sources or precursors of HNO_3 and H_2SO_4 are compiled in Table 1. The range of estimates in the literature is large, especially for the natural sources, which cannot be derived from economic data as can the anthropogenic sources. The volcanic contribution is, of course, subject to extreme fluctuation. During the time period covered by our samples, the volcanic activity was low except for the years 1902, 1912 and 1963¹⁵. With the exception of the already mentioned Katmai explosion, all large eruptions were in the equatorial zone. Their contribution to the sulphate level in our samples is not distinguishable from the scatter.

Inputs from land-based sources to the impurity level in Greenland must travel quite far and must be able to survive in the atmospheric boundary layer, where removal by precipitation is very effective. Sources in the free troposphere or in the ocean surrounding Greenland will contribute more easily to the concentration in the precipitation of South Greenland. Essentially all anthropogenic sources are land-based with the exception of jet airplanes.

Using the estimated source strengths of the different NO_3^- and SO_4^{2-} precursors, one can calculate the percentage of the '1900 level' of $[\text{NO}_3^-]$ and $[\text{SO}_4^{2-}]$ that is derived from land-based sources. Our calculation assumes that the different sources contribute linearly to the impurity level in the snow and are split into continental (land-based) and other (non-land-based) sources. No interhemispherical mixing is assumed. Two time periods, 1895-1901 and 1973-78, were considered, assuming the emission rates indicated in Table 1 and using the mean measured concentration of these time intervals.

The '1900 level' may be expressed as $\gamma P_C + \delta P_O = C_{1900}$; the 'recent level' as $\gamma(P_C + P_A) + \delta P_O = C_{\text{recent}}$, where γ and δ are factors linking emission rates and firm concentration; P_C , P_A and P_O are the land-based (continental), anthropogenic (~1975) and non-land-based (ocean) production rates, respectively; and C_{1900} and C_{recent} are the '1900' and 'recent' firm concentrations, respectively.

This model is no more than a first approximation. Natural

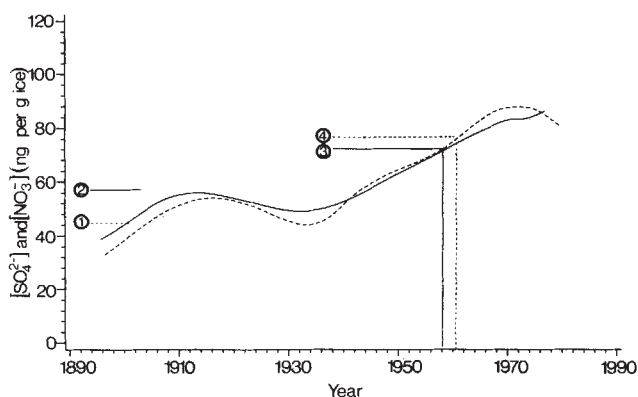


Fig. 3 Time development of atmospheric load with nitrate (—) and sulphate (---). Comparison of anthropogenic and natural input. (1) '1900' $[\text{SO}_4^{2-}]$ (+1 σ). (2) '1900' $[\text{NO}_3^-]$ (+1 σ). (3) '1900' $[\text{NO}_3^-]$ (+1 σ) + 16 p.p.b. ($\approx 11 \text{ Tg N yr}^{-1}$ anthropogenic emission) for when anthropogenic N sources were approximately equal to global natural N sources. (4) '1900' $[\text{SO}_4^{2-}]$ (+1 σ) + 28 p.p.b. ($\approx 57 \text{ Tg S yr}^{-1}$ anthropogenic emission) for when anthropogenic S sources were approximately equal to global natural S sources.

sources are diffuse, whereas the anthropogenic components have more the character of point sources. The results of this model calculation give for the 1900 level a contribution of land-based sources to the concentration in the ice of 54% for sulphate and 37% for nitrate. It should be pointed out that this number depends strongly on the emission rates that we have selected in Table 1.

Based on our calculation, 1 Tg yr^{-1} of land-based sulphur source corresponds in the mean annual concentration of the snow in Dye 3 to an additional $[\text{SO}_4^{2-}]$ of $\sim 0.6 \text{ p.p.b.}$, whereas 1 Tg yr^{-1} of land-based nitrogen source corresponds to $\sim 1.4 \text{ p.p.b.}$ of additional $[\text{NO}_3^-]$, respectively. These numbers depend only weakly on the assumed emission rates. Thus, the source distribution and the long-range transport of the sulphur and nitrogen components seem to be different. Our data suggest that the nitrogen component is more easily transported through the boundary layer than is the sulphur component, which seems to be contrary to the calculation published by Rhode *et al.*¹⁶. However, their calculation was based on precipitation data from the European Air Chemistry Network, whereas our data apply to Greenland. The low proportion of the land-based contribution to the pre-industrial $[\text{NO}_3^-]$ supports the idea that NO_x from the stratosphere and produced by lightning are the main sources of the background nitrate in remote regions^{6,17}. Such sources in the free troposphere were also postulated by Huebert and Lazrus¹⁸ to explain their $[\text{HNO}_3]$ measurements in the free troposphere.

Our sulphate concentration in the period 1895-1904 is higher than the values given by Herron⁷ for earlier periods. Because volcanic activity was low during 1895-1904, our higher measured concentration indicates that by this time the sulphate level was already considerably elevated over pre-industrial values, possibly reflecting early industrial activity. Our model is inconsistent with the pre-industrial sulphate level given by Herron (22 p.p.b.) and would require a land contribution of over 100%. The most probable explanation is that the natural land-based source used in our model is an overestimate.

Using the relation given above between concentration increase in the ice and source strength, we can deduce the time when anthropogenic sources surpassed the natural source concentration given in Table 1. Figure 3, illustrating this behaviour, shows that the anthropogenic sources started to dominate the total natural sources around 1960. This agrees with the anthropogenic emission data for sulphate given by Möller¹⁹.

Our data suggest that man-made pollution had already started to affect remote regions more than 30 yr ago. The nitrate and sulphate concentrations in Greenland contain information which can ultimately be used to interpret the development and

Table 1 Global emission ranges

		Northern Hemisphere range (Tg S yr ⁻¹)	Southern Hemisphere range (Tg S yr ⁻¹)
Sulphur source			
Natural:	Biogenic sea	14-32	18-40
	Biogenic land	2-74 (32)*	1-36
	Volcanoes	0.6-6 (3)*	0.4-4
Man-made: (1976)		69-98 (98)*	5-6
Nitrogen source			
Natural:	Soil	0.7-7 (4)†	0.3-3
	Wildfire	0.2-0.6 (0.4)†	0.1-0.3
	Ox. NH_3	0-3 (1.9)†	0-2
	Biomass burn.	3-8 (5.5)†	
	Lightning	1-4	1-4
	Stratosphere	0.15-0.45	0.1-0.45
Man-made:	Fossil fuel	7-17 (13)†	0.5-1
	Biomass burn.	6-40 (18)†	6-18
	Jets	0.2-0.4 (0.3)†	<0.1

Data compiled from refs 19-24.

* Assumed production for calculations: natural production land-based, 35 Tg yr^{-1} (=total land-based production pre-industrial); natural and man-made production land-based, 133 Tg yr^{-1} .

† Assumed production for calculations: natural production land-based, 11.4 Tg yr^{-1} (=total land-based production pre-industrial); natural and man-made production land-based, 43 Tg yr^{-1} .

distribution of sources of acid rain, although at the moment our understanding of source strengths and transport is still so rudimentary that the interpretation must remain largely qualitative. More widespread drilling of firn cores in polar regions would allow the historical development of the acid deposition pattern to be established and sophisticated source receptor models to be tested. Nevertheless, the data already show that human activity drastically changed the atmospheric load of the acidic trace species HNO_3 and H_2SO_4 . This fact emerges more clearly in the historical record from remote locations, such as Dye 3, than from direct measurements in populated areas which are influenced by changes in measuring technique and changed local distribution of pollution with time.

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1. Hidy, G. M. *et al.* *Air Pollution Control Ass. J.* **31**, 333-354 (1984).
2. Likens, G. E. *Scient. Am.* **241** (4), 43-51 (1979).
3. Marsh, A. R. W. *Atmos. Envir.* **12**, 401-406 (1978).
4. Charlson, R. J. & Rhode, H. *Nature* **295**, 683-685 (1982).
5. Beer, J. *et al.* *Radiocarbon* **25**, 269-278 (1983).
6. Neftel, A. *et al.* *Greenland Ice Sheet Program*, Philadelphia (Am. geophys. Un. Monogr. 1984).
7. Herron, M. M. *J. geophys. Res.* **87**, 3052-3060 (1982).
8. Rasmussen, K. *et al.* *Icarus* **58**, 101-108 (1984).
9. Herron, M. M. *Am. chem. Soc. Symp. Ser.* **176**, 303-318 (1982).
10. Turco, R. P. *et al.* *Science* **214**, 19-23 (1981).
11. Legrand, M. & Delmas, R. J. *Atmos. Envir.* **18** (9), 1867-1874 (1984).
12. Hammer, C. U. *J. phys. Chem.* **87**, 4099-4103 (1983).
13. Delmas, R. J. *et al.* *J. geophys. Res.* **87**, 4314-4318 (1982).
14. Chameides, W. L. & Davis, D. *Chem. Engng News* **60**, 38-52 (1982).
15. Lamb, H. H. *Phil. Trans. R. Soc. A266*, 425-533 (1970).
16. Rhode, H. *et al.* *Tellus* **33**, 132-141 (1981).
17. Hameed, S. *et al.* *Geophys. Res. Lett.* **8**, 591-594 (1981).
18. Huebert, B. J. & Lazrus, A. L. *J. geophys. Res.* **85**, 7322-7328 (1980).
19. M  ller, D. *Atmos. Envir.* **18**, 29-36 (1984).
20. Hahn, J. & Crutzen, P. J. *Phil. Trans. R. Soc. B296*, 521-541 (1982).
21. Ehhalt, D. H. & Drummond, J. W. in *Chemistry of Unpolluted and Polluted Troposphere* (eds Georgii, H. W. & Jaeschke, W.) 119-151 (Reidel, Dordrecht, 1982).
22. Cullis, C. F. & Hirschler, M. M. *Atmos. Envir.* **14**, 1263-1278 (1980).
23. M  ller, D. *Atmos. Envir.* **18**, 19-27 (1984).
24. Nguyen, B. C. *et al.* *J. geophys. Res.* **88**, 10903-10914 (1983).

Late Eocene microtektites and radiolarian extinctions on Barbados

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A late Eocene microtektite layer has been found in three exposed sections on Barbados, West Indies. These microtektites and associated tektite fragments are compositionally similar to the North American tektites and thus, like the Caribbean and Gulf of Mexico microtektites, may belong to the strewn field of 'North American' microtektites which have been found in deep-sea deposits from the Caribbean Sea, across the Pacific and into the northeastern Indian Ocean^{1,2}. The 'North American' layer has been reported to be approximately synchronous with several radiolarian extinctions^{3,4} and with an anomaly in iridium concentrations⁵⁻⁷, and it has been hypothesized that the microtektites, the extinctions and the iridium anomaly are all related to an asteroid impact. We report here that the radiolarian extinctions predate the microtektite layer and that the highest Ir concentrations apparently coincide with the extinctions. Thus, although the extinctions may be causally related to the Ir anomaly, the microtektites appear not to be.

Table 1 Major oxide composition of selected Barbados microtektites and tektite fragments and North American tektites

Sample no.	SiO ₂	Al ₂ O ₃	FeO	MgO	CaO	Na ₂ O	K ₂ O	TiO ₂
Barbados microtektites								
179-16-2	84.2	10.4	1.48	0.45	0.20	0.60	1.77	0.49
176-16-14	79.4	11.7	3.23	0.61	0.35	1.28	2.54	0.54
173F	78.6	13.0	3.15	0.99	0.63	0.73	1.88	0.61
173E	77.5	12.3	3.67	0.90	0.86	0.83	2.86	0.77
179-16-5	76.0	13.5	3.68	0.96	0.78	1.23	2.78	0.64
Barbados tektite fragments								
173G	81.7	10.2	2.06	0.41	0.94	1.63	2.53	0.43
173H	81.0	10.3	2.71	0.49	0.73	1.56	2.56	0.43
181A	78.9	12.4	3.28	0.56	0.17	1.41	2.04	0.81
173C	78.8	12.6	3.20	0.54	0.46	1.34	2.09	0.60
173D	78.6	12.8	3.18	0.52	0.50	1.31	2.04	0.70
North American tektites								
BM	81.31	10.96	2.43	0.53	0.50	1.50	2.17	0.53
181D	81.0	11.0	2.58	0.60	0.36	0.84	2.61	0.52
181C	78.4	12.5	3.22	0.62	0.41	1.42	2.25	0.66
B-50	78.19	12.35	3.50	0.73	0.65	1.71	2.23	0.74
B-6	76.25	13.49	3.98	0.74	0.74	1.63	2.22	0.74

The data for North American tektite samples BM, B-50 and B-6 are from ref. 14. The rest of the data were obtained by energy dispersive X-ray analysis. A total of 18 microtektites and five tektite fragments from Bath Cliff were analysed.

Microtektites belonging to two Quaternary tektite-strewn fields on land, the Australasian and Ivory Coast, have been found in deep-sea sediment cores taken in the Australasian/Indonesian region and off the Ivory Coast, respectively¹. Much older than the Quaternary group are the late-Eocene North American microtektites, found in a low-latitude band stretching from the Caribbean to the northeastern Indian Ocean. The glassy rounded tektites and microtektites are believed to be produced by impacts^{8,9}. Correlation of late-Eocene microtektites, anomalous Ir, and radiolarian extinctions^{6,7} has been used to suggest a relationship between impacts and extinctions similar to that which has been inferred for the Cretaceous-Tertiary event¹⁰.

Recent detailed biostratigraphical investigations on Barbados¹¹ provided the possibility of locating, on land, the stratigraphical level of occurrence of the microtektites, which had previously been available in only very limited amounts, at scattered localities, in deep-sea cores. The stratigraphical resolution attainable in these land-based sections is 2-10 times greater than that attained in deep-sea sediment cores containing the microtektite layer. For example, the stratigraphical interval separating the earliest occurrence of the radiolarian *Thyrocyclis bromia* and the evolutionary transition of *Lithocyclia aristotelis* to *L. angusta* is 100-110 m at Bath Cliff, 10-20 m at Deep Sea Drilling Project Site 149 (ref. 12), about 10 m at DSDP Site 543 (unpublished data) and 15-45 m at DSDP Site 94 (ref. 13). The microtektites were found in this interval at a single stratigraphical level (10-30 cm thick) in three sections; Bath Cliff, Gay's Cove North and Gay's Cove South.

All three sections were sampled continuously, by taking contiguous samples 5 cm thick. This provided a basis for examining changes in the microtektites throughout their occurrence, and details of the abundances in the uppermost parts of the ranges of radiolarian species as they became extinct. Weighed subsamples of the channel samples were disaggregated and sieved to obtain the >63-μm fraction for investigation of the radiolarians and the microtektites.

Identification of the Barbadian microtektites and tektite fragments as belonging to the late-Eocene horizon which has been called the 'North American' strewn field is indicated by their late Eocene age and by their chemical composition (Table 1). North American tektites are characterized by low lime and magnesia content as compared with tektites from other strewn fields¹⁴, and this is reflected in the composition of the Barbados microtektites and tektite fragments. They have a unimodal size distribution, this being more pronounced in the samples near

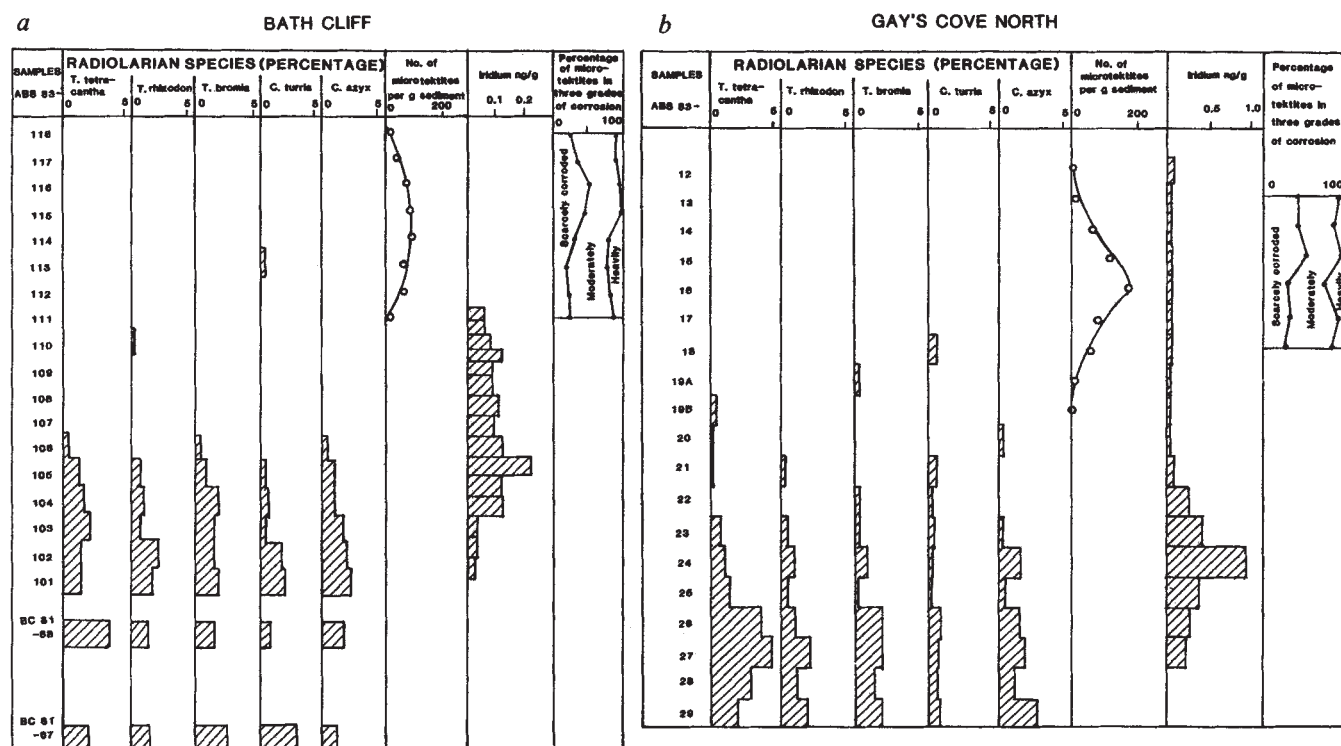


Fig. 1 The upper limits, in the Bath Cliff (a) and Gay's Cove North (b) sections, of species that became extinct slightly below the microtektite layer. Abundances are given as percentages of the total assemblage coarser than 63 μm , estimated from assemblages of 6,000–26,000 radiolarians from each sample. Although the five radiolarian species plotted constitute a high proportion of the 20 or so commonly used for late-Eocene/early-Oligocene stratigraphy, the proportion is less remarkable when considered in relation to the 200–300 additional species present in these assemblages but not used stratigraphically. In the right-hand columns are numbers of microtektites and their degree of corrosion, and Ir concentrations. In each section, the extinctions clearly predate the microtektite event, and coincide with the high Ir concentrations.

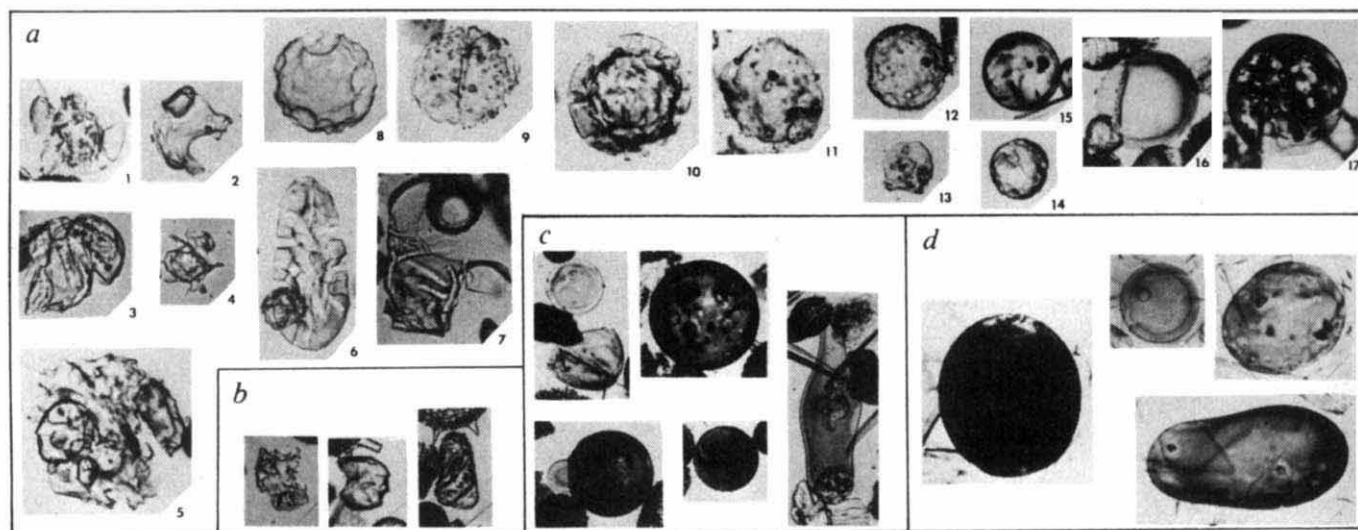


Fig. 2 Representative examples of microtektites from: a, Barbados; b, DSDP Site 543; c, DSDP Site 149; and d, DSDP Site 94. In a, specimens 15–17 were counted as smooth, regular; specimens 8–14 as moderate; and 1–7 as highly irregular. Magnification $\times 105$.

the middle of the layer than towards its limits. The 80–100- μm and 100–120- μm size grades contain more specimens than do the other 20- μm increments, but the greatest volume of material is in the slightly larger size grades. Assuming a grain bulk density of 2.5 g cm^{-3} for both the sediment and the tektite material and an average bulk dry density of 1.32 g cm^{-3} for the sediment¹¹, the net fluence of microtektites and tektite fragments is 45 tonnes km^{-2} at the Bath Cliff section. Although the thicknesses of the layer at Gay's Cove North and Gay's Cove South is less certain because of imprecise relations between the line of samp-

ling and the attitude of the beds (resulting from local small-scale tectonic complications), the total tektite contribution appears to be greater, perhaps as much as 80 tonnes km^{-2} in the former section and 100 tonnes km^{-2} at the latter.

This is substantially less than the 1,000 tonnes km^{-2} calculated for Caribbean and Gulf of Mexico sites³. The lower figures for Barbados may indicate that this site is closer to the edge of the strewn field. Another factor is that the Barbados microtektites are now above sea level and have probably experienced more solution than the microtektites from the submarine localities.

In an attempt to detect the iridium anomaly reported just below the main microtektite layer by Ganapathy⁶, and coincident with it by Alvarez *et al.*⁷, neutron activation analysis was performed on a continuous series of Bath Cliff samples from about 5 cm above the base of the layer to 25 cm stratigraphically below it, and on a continuous series of Gay's Cove North samples from about 15 cm stratigraphically above the microtektite peak to about 30 cm below it. The results show a peak in Ir concentration well below the microtektite layer and at practically the same level as the radiolarian extinctions in both sections (Fig. 1a, b), but it represents a substantially lower net fluence of Ir ($<2 \text{ ng cm}^{-2}$ at Bath Cliff and $\sim 7 \text{ ng cm}^{-2}$ at Gay's Cove North) than would be expected on the basis of results obtained by Ganapathy⁶ (63 ng cm^{-2}). Our data, like Ganapathy's, would indicate that the anomalous Ir is not coincident with the North American microtektite layer, but we cannot rule out a relationship between the radiolarian extinctions and another impact event. Clinopyroxene-bearing glass spherules (as distinct from the microtektites) are coincident with the Ir anomaly in core RC9-58 (refs 6, 15). These may have once existed at the level at which we found the Ir, and have since been dissolved by ground water above sea level.

The radiolarian extinctions clearly predate the microtektite layer in these sections (Fig. 1). Their apparent synchronicity in sections investigated earlier may be due to much lower rates of sediment accumulation and thus lower stratigraphical resolution. The tailing-upward of the radiolarian species could be attributable to bioturbation¹⁶ (and the times of their extinction pinpointed at slightly above the levels at which their abundances begin markedly to decline). However, bioturbation does not provide an explanation for the separation between the microtektites and the extinctions. At Bath Cliff, the separation of $\sim 27 \text{ cm}$ probably represents an interval of 13,000–14,000 yr. This is based on an estimated sediment accumulation rate of $\sim 20 \text{ m Myr}^{-1}$ (calculated from separation of biostratigraphical events)^{11,17}. This contradicts any causal relationship between the extinctions and the late-Eocene microtektite event^{6,7}.

Although compositional similarities and stratigraphical position imply a correlation between the Barbadian microtektites and the North American field, there are differences that may reflect variations within the field. Texturally, these samples range from regular spheres and teardrops to highly corroded or angular fragments. The proportion of fragments appears to be much greater in Barbados and at DSDP Site 543 (generally $>60\%$, Fig. 2a, b) than in the Caribbean and Gulf of Mexico sites ($<8\%$ (ref. 4); Fig. 2c, d). Moreover, the Barbadian microtektites have a slightly higher SiO_2 content and have a generally paler colour than those from the Caribbean and Gulf of Mexico. Keller *et al.*¹⁸ have suggested that there may be multiple late-Eocene microtektite layers, not associated with profound changes in the biota, but this cannot be evaluated here. The basic question of the multiple falls will ultimately be resolved by careful description of the physical, chemical and isotope characteristics of the microtektites at each of their occurrences and by their placement in a more precise biostratigraphical framework^{19,20}.

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- Glass, B. P., Swinicki, M. B. & Zwart, P. A. *Proc. 10th Lunar planet. Sci. Conf.* 2535–2545 (1979).
- Glass, B. P. & Crosbie, J. R. *Am. Ass. petrol. Geol. Bull.* **66**, 471–476 (1982).
- Maurrasse, F. & Glass, B. *Trans. VII Conf. Géol. Caraïbes* 205–212 (1976).
- Glass, B. P. & Zwart, M. J. *Bull. geol. Soc. Am.* **90**, 595–602 (1979).
- Asaro, F., Alvarez, L. W., Alvarez, W. & Michel, H. V. *Geol. Soc. Am. Spec. Pap.* No. 190, 517–528 (1982).
- Ganapathy, R. *Science* **216**, 885–886 (1982).
- Alvarez, W., Asaro, F., Michel, H. V. & Alvarez, L. W. *Science* **216**, 886–888 (1982).
- Barnes, V. E. & Barnes, M. A. *Tektites* (Dowden, Hutchinson and Ross, Stroudsburg, 1973).

- Shaw, H. F. & Wasserburg, G. J. *Earth planet. Sci. Lett.* **60**, 155–177 (1982).
- Alvarez, L. W., Alvarez, W., Asaro, F. & Michel, H. V. *Science* **208**, 1095–1108 (1980).
- Saunders, J. B. *et al. Micropaleontology* **30**, 390–425 (1985).
- Riedel, W. R. & Sanfilippo, A. in *Init. Rep. DSDP* **15**, 705–751 (1973).
- Sanfilippo, A. & Riedel, W. R. *Init. Rep. DSDP* **10**, 475–611 (1973).
- Chao, E. C. T. in *Tektites* (ed. O'Keefe, J. A.) 51–94 (University of Chicago Press, 1963).
- Glass, B. P., DuBois, D. L. & Ganapathy, R. *Proc. 13th Lunar planet. Sci. Conf.* **1**, A425–A428 (1982).
- Ruddiman, W. F. *et al. Sedim. Geol.* **25**, 257–276 (1980).
- Berggren, W. A., Kent, D. V. & Flynn, J. J. *Geol. Soc. Lond. Spec. Pap.* (in the press).
- Keller, G., D'Hondt, S. & Vallier, T. L. *Science* **221**, 150–152 (1983).
- Glass, B. P. *Science* **224**, 309 (1984).
- Keller, G., D'Hondt, S. & Vallier, T. L. *Science* **224**, 309–310 (1984).

Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins

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Acquiring the ability to regulate morphogenesis in plants has long been an elusive goal. Previous research has established that 'thin cell-layer' explants from the surface of the floral branches of tobacco can be induced to form either callus, vegetative buds, flowers or roots by adjusting the pH and the ratio of auxin to cytokinin in the culture medium^{1–5}. We now report that oligosaccharins (oligosaccharides with regulatory activity) derived from plant cell walls can, at concentrations of $\sim 10^{-8}$ or 10^{-9} M , induce tobacco explants to form vegetative buds instead of flowers or callus, or flowers instead of vegetative buds. Other oligosaccharins can induce the explants to form roots instead of vegetative buds. This ability of oligosaccharins to control morphogenesis suggests that these fragments are *in situ* regulators of morphogenesis.

Tissue cultures of some plant species have been induced to form vegetative buds and/or roots by varying the relative concentration of growth regulators (for example, auxin and cytokinin)¹. Despite extensive trials with various concentrations of growth regulators, carbohydrates and mineral salts, however, it remains difficult to induce tissue cultures of many plant species to form organs. Tissue cultures of those plant species that can be induced by means of varying the auxin/cytokinin ratio usually form only vegetative buds, roots, or undifferentiated callus. Besides altering the concentration of these two growth regulators, adding sucrose or glucose^{2,3} and varying the pH of the culture medium^{4,5} are two other factors that have been shown to affect organ formation in tobacco tissue culture. Moreover, cultured tissues with organized cells (for example, epidermis, subepidermis) may have greatly increased capacities to form organs^{6–10}. The ability to control morphogenesis in tissues has been particularly well developed in 'thin cell-layer' explants of tobacco^{9,11,12}. We have used this system for our experiments.

The tobacco explants are obtained by excising cortical tissue of floral branches in $1 \times 10\text{-mm}$ strips containing epidermis (1 cell layer), subepidermis (1 cell layer) and parenchyma (1–3 cell layers). When these thin cell-layer strips, or 'tobacco explants' as we will call them, are floated on liquid culture media containing salts, glucose (as an energy source), auxin (indolebutyric acid) and cytokinin (kinetin), and when the pH of the culture medium is adjusted to a specific value, the explants are induced to form flowers, vegetative buds, roots or undifferentiated callus. Thus, exogenously applied factors determine the specific type of organ formed by the explants.

Table 1 Effect of EPG fragments on morphogenesis of tobacco explants

	Concentration of EPG fragments ($\mu\text{g ml}^{-1}$)	Flowers	Vegetative buds	Callus formation
Medium I pH 3.8	0	600*	0	0
	1	100	1,800	0
Medium II pH 5.0	0	0	0	100%
	1	0	400	0
	10	0	1,200	0
pH 6.0	0	0	1,800	0
	1	600	18	0

* Total number of organs (flowers, floral shoots, vegetative buds or roots) formed or percentage of explants exhibiting callus formation (cell proliferation without organogenesis) in three separate experiments. The numbers are the sums in each experiment of the organs on 20 explants.

Methods. Tobacco plants were cultured at 24 °C under a 16-h photoperiod of natural light, complemented by artificial light (25 W m⁻²) when the natural light was < 50 W m⁻². The artificial light was a mixture of fluorescent (Mazda-type 65 W) and incandescent (60 W). Relative humidity was maintained at 65%. The tobacco explants were excised from basal parts of floral branches of *Nicotiana tabacum* L. c.v. samsun when the inflorescence contained 20 green fruits. The terminal green fruit of the cyme was 2.0–2.3 cm long and ~1.2 cm in diameter. The explants were obtained from floral branches that had been surface-sterilized (7% Ca(OCl)₂ for 10 min) and then rinsed three times in sterile water. Twenty explants were floated on liquid medium (40 ml) in Petri dishes (10 cm). The dishes were sealed with Parafilm. The basal medium was composed of Murashige and Skoog's macro- and micro-elements²⁹, myo-inositol (100 mg l⁻¹), thiamine-HCl (0.4 mg l⁻¹) and glucose (30 g l⁻¹). Kinetin was added at 5 × 10⁻⁷ M and indolebutyric acid (IBA) at 5 × 10⁻⁷ M in medium I and at 3 × 10⁻⁶ M in medium II. Each medium was adjusted to the specified pH with either HCl or KOH. Oligosaccharin mixtures (1 or 10 $\mu\text{g ml}^{-1}$) were added (the addition of the oligosaccharins did not alter the pH of the media). Each medium was sterilized by passing it through a Millipore filter (0.2 μm). The explants were incubated at 24 °C and under continuous light (20 W m⁻²). After 30 days, the morphogenesis of the explants was assessed. Each experiment was repeated three or four times. The results presented here are a summary of the data collected in all of these experiments. Two different mixtures of sycamore cell wall fragments (oligosaccharins) were used. One set of oligosaccharins (EPG fragments) was released by endo- α -1,4-polygalacturonase treatment of isolated sycamore cell walls. Endopolygalacturonase was purified as described elsewhere³⁰, as were the cell walls³¹. The cell walls were treated with the enzyme and the solubilized fragments were isolated as described elsewhere³², except that the pH of the fragments was adjusted to 6.5 before the final exhaustive dialysis against deionized water. The second mixture of sycamore cell wall fragments was prepared by partial base-catalysed solubilization and partial degradation of cell wall components. These B fragments were prepared as follows: 4 MKOH (500 ml), containing NaBH₄ (500 mg) was added to isolated sycamore cell walls (5 g). The suspension was stirred overnight and then passed through two layers of Whatman GF/A filter paper. While being vigorously stirred, the soluble material was adjusted to pH 6.5 with concentrated HCl. The solvent was evaporated at 40 °C under reduced pressure. The supernatant solution was decanted repeatedly from salt crystals that formed in the evaporation flask. The salt crystals were washed with a small volume of distilled water and the washes were added to the supernatant. Once the volume of the supernatant had been reduced to < 80 ml, the preparation was desalted on a large Sephadex G-10 column (~600 ml bed volume) eluted with distilled water. The carbohydrate-positive fractions (determined by the anthrone colorimetric assay³³) with low conductivity (low salt) were pooled and then dried under reduced pressure at 40 °C. Methanol (200 ml) was added to the residue and evaporated, twice. Then methanol containing 10% acetic acid (300 ml) was added and dried and this procedure was repeated three times. Finally, methanol (200 ml) was added and the residue was dried three times. These evaporations were performed to volatilize the methyl ester of borate. The residue was dissolved in distilled water, the pH adjusted to 7.0 and the solution lyophilized.

It would be difficult to study the biochemical and molecular genetic phenomena underlying induced morphogenesis in these tissues because auxin and cytokinin are both pleiotropic growth regulators; that is, they have many effects on plant tissues besides influencing morphogenesis. Furthermore, altering the pH of the culture medium is also likely to have effects on such diverse factors as the biochemical properties of the cell wall and the ability of the plasma membrane to perceive and transport plant growth regulators^{13,14}, salts¹⁵ and other metabolites^{16,17}. We hypothesize that auxin, cytokinin and the pH of the culture medium affect the release of, processing of and/or responsiveness to chemical messages, each of which is not broadly pleiotropic and each of which regulates a delineated set of biochemical processes that regulate morphogenesis^{4,18–20}. The experiments described here were designed to obtain evidence for the existence of chemical messages that have more specific effects than do the classical plant growth regulators.

Certain oligosaccharides, derived by means of fragmenting plant cell wall polysaccharides, are chemical messages that have been shown to be capable of regulating physiological processes in plants (for example, refs 18, 21–26 and S. Doares, A.G.D. and P.A., unpublished results). Oligosaccharides capable of regulating physiological processes have been called oligosaccharins²⁷. The discovery that oligosaccharins can control gene expression in plants motivated us to design the present experiments (see Table 1, methods), which demonstrate that oligosaccharins derived from the cell walls of suspension-cultured sycamore cells can regulate the morphogenesis of the thin cell-layer explants of tobacco.

The tobacco explants, when grown in medium I at pH 3.8, formed an average of 10 flowers each (Table 1). Adding 1 $\mu\text{g ml}^{-1}$ of EPG fragments (oligosaccharins released by endo- α -1,4-polygalacturonase treatment of sycamore cell walls) inhibited flowering by 87% and induced the explants to form many vegetative buds over the entire surface area. (Flowers formed only on the basipetal end of the explants.) In medium II at pH 5.0, in the absence of added EPG fragments, the explants developed, not organs, but rather, large amounts of undifferentiated callus. The formation of callus was inhibited by 1 or 10 $\mu\text{g ml}^{-1}$ of EPG fragments and the formation of large numbers of vegetative buds was induced by EPG fragments. In medium II at pH 6.0, in the absence of added fragments, the explants formed vegetative buds. However, formation of buds was almost completely inhibited and, instead, formation of flowers was induced by adding 1 $\mu\text{g ml}^{-1}$ of EPG fragments.

The base-solubilized (B) fragments have an effect similar to that of the EPG fragments in medium I at pH 3.8 (Table 2). Although the B fragments did induce formation of vegetative buds and inhibit flowering, 10 $\mu\text{g ml}^{-1}$ of the B fragments was required, compared with the 1 $\mu\text{g ml}^{-1}$ of EPG fragments required in the previous example. Furthermore, B fragments caused the explants to form floral shoots, rather than flowers, on the same explants that formed vegetative buds. In medium II at pH 6.0, these B fragments, like the EPG fragments, inhibited vegetative bud formation; but the B fragments induced roots, rather than flowers, to form.

Table 2 Effect of B fragments on morphogenesis of tobacco explants

	Concentration of B fragments ($\mu\text{g ml}^{-1}$)	Flowers	Floral shoots	Vegetative buds	Roots	Callus
Medium I pH 3.8	0	600*	0	0	0	0
	1	600	300	27	0	0
	10	0	300	1,200	0	0
Medium II pH 5.0	0	0	0	0	0	100%
	1	0	0	0	300	100%
	10	0	0	0	420	100%
pH 6.0	0	0	0	1,800	0	0
	1	0	0	0	420	100%
	10	0	0	0	450	100%

* See Table 1 legend.

We estimate the concentration of active oligosaccharins in the EPG and B fragment mixtures to be 10^{-8} or 10^{-9} M, which matches the specific activity observed for other oligosaccharins^{22,28}. Also, like the other oligosaccharins²¹, the EPG and B fragments exhibit activity over a rather narrow (one or two orders of magnitude) range of concentration; the morphogenetic effects disappear when the concentration is too high or too low (unpublished data). Furthermore, the EPG and B fragments exhibit the described effects in the presence of 30 mg ml^{-1} of glucose.

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1. Skoog, F. & Miller, C. O. *Symp. exp. Biol.* **15**, 118-131 (1957).
2. Cousson, A. & Tran Thanh Van, K. *Pl. Physiol.* **72**, 33-36 (1983).
3. Tran Thanh Van, K. in *Regulation of Morphogenesis in Plant Tissue Culture and its Biotechnological Application* (eds Barz, W., Reinhard, E. & Zenk, M. H.) 367-385 (Springer, Berlin, 1977).
4. Cousson, A. & Tran Thanh Van, K. *Physiol. Pl.* **51**, 77-84 (1981).
5. Cousson, A., Toubart, P. & Tran Thanh Van, K. *Bull. Soc. bot. Fr.* (in the press).
6. Aghion-Pratt, D. *Physiol. Vég.* **3**, 229-303 (1965).
7. Chlyah, H. *Pl. Physiol.* **54**, 341-348 (1974).
8. Chlyah, H. *Pl. Physiol.* **62**, 482-485 (1978).
9. Tran Thanh Van, M. *Planta* **115**, 87-92 (1973).
10. Tran Thanh Van, K. *Int. Rev. Cytol. Suppl.* **11A**, 175-194 (1980).
11. Tran Thanh Van, K. *A. Rev. Pl. Physiol.* **32**, 291-311 (1981).
12. Tran Thanh Van, K., Dien, D. T. & Chlyah, A. *Planta* **119**, 149-159 (1974).
13. Rubery, P. H. & Sheldrake, A. R. *Nature new Biol.* **224**, 285-288 (1973).
14. Rubery, P. H. & Sheldrake, A. R. *Planta* **118**, 101-121 (1974).
15. Martin, S. M. & Rose, D. *Can. J. Bot.* **54**, 1264-1270 (1976).
16. Colombo, R., De Michaelis, M. I. & Lado, P. *Planta* **138**, 249-256 (1978).
17. Komor, E. & Tanner, W. *Biochim. biophys. Acta* **241**, 170-179 (1971).

Thus the effect of the EPG and B fragments on morphogenesis does not result from a nonspecific carbohydrate effect. The ability of oligosaccharins to regulate morphogenesis in tobacco explants makes apparent the possibility that these fragments are *in situ* regulators of morphogenesis. Our goal is to ascertain whether this is, or is not, the case.

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18. Darvill, A. G. & Albersheim, P. A. *Rev. Pl. Physiol.* **35**, 243-275 (1984).
19. Darvill, A. G. *et al. J. Sci. Suppl.* (in the press).
20. McNeil, M., Darvill, A. G., Fry, S. C. & Albersheim, P. A. *Rev. Biochem.* **53**, 625-663 (1984).
21. Gollin, D. J., Darvill, A. G. & Albersheim, P. *Biol. Cell* **51**(2), (in the press).
22. York, W. S., Darvill, A. G. & Albersheim, P. *Pl. Physiol.* **75**, 295-297 (1984).
23. Nothnagel, E. A., McNeil, M., Albersheim, P. & Dell, A. *Pl. Physiol.* **71**, 916-926 (1983).
24. Jin, D. J. & West, C. A. *Pl. Physiol.* **74**, 989-992 (1984).
25. Fry, S. C., Darvill, A. G. & Albersheim, P. *Br. Pl. Growth Regulator Group Monogr.* **9**, 33-43 (1983).
26. Yamazaki, N., Fry, S. C., Darvill, A. G. & Albersheim, P. *Pl. Physiol.* **72**, 864-869 (1983).
27. Albersheim, P. *et al. in Structure and Function of Plant Genomes* (eds Ciferri, O. & Dure, L. III) 293-312 (Plenum, New York, 1983).
28. Sharp, J. K., Valent, B. & Albersheim, P. *J. Biol. Chem.* **259**(18), 11312-11320 (1984).
29. Murashige, T. & Skoog, F. *Physiologia Pl.* **15**, 473-497 (1962).
30. English, P. D., Maglothlin, A., Keegstra, K. & Albersheim, P. *Pl. Physiol.* **49**, 293-297 (1972).
31. Talmadge, K. W., Keegstra, K., Bauer, W. D. & Albersheim, P. *Pl. Physiol.* **51**, 158-173 (1973).
32. Darvill, A. G., McNeil, M. & Albersheim, P. *Pl. Physiol.* **62**, 418-422 (1978).
33. Dische, Z. in *Methods in Carbohydrate Chemistry* Vol. 1 (eds Whistler, R. L. & Wolfrom, M. L.) 478-481 (Academic, New York, 1962).

Assembly in *E. coli* of a functional multi-subunit ribulose biphosphate carboxylase from a blue-green alga

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The enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBPCase) has a pivotal role in photosynthetic organisms, catalysing the first reactions of the interlocked but opposing pathways of photosynthesis and photorespiration^{1,2}. The agricultural interest in RuBPCase centres on the possibility of altering the structure of the enzyme to improve net photosynthetic yield, but such studies are hampered by the structural complexity of the hexadecameric holoenzyme in plants which prevents *in vitro* dissociation and reconstitution, apparently due to the properties of the large subunits^{3,4}. We have chosen to express the large and small subunit genes of the cyanobacterium *Synechococcus* PCC6301 in *Escherichia coli* to develop an experimental system in which structural changes introduced into RuBPCase polypeptides could be assessed following subunit assembly. The cyanobacteria (blue-green algae) are autotrophic prokaryotes which have a plant-like photosynthetic mechanism and RuBPCase structure^{5,6}. We report here the synthesis of an active RuBPCase in *E. coli* which contains both large and small subunit polypeptides.

The genes for the large subunit (L) and small subunit (S) of RuBPCase from *Synechococcus* have been identified on a 2.17-kilobase (kb) *Pst*I fragment of DNA^{7,8}. To obtain expression of both L and S genes, this *Pst*I fragment was cloned into the *Pst*I site of the plasmid pLa2311 (ref. 9) downstream from a λ *P_L* promoter, to yield plasmid pSV55 (Fig. 1a). To demonstrate that RuBPCase polypeptides could be synthesized following derepression of *P_L*, a culture of *E. coli* cells containing pSV55 was incubated at 41 °C and a protein extract prepared. Electrophoresis of the total cell proteins in polyacrylamide gels

containing SDS, followed by Western blotting using anti-wheat RuBPCase serum, revealed the presence of two immunoreacting polypeptides (Fig. 1b, lane 2). The smaller polypeptide has a relative molecular mass (M_r) of 52,000 and co-migrates with L present in maize leaf extracts (Fig. 1b, lane 3). The larger polypeptide of M_r 70,000-72,000 is a fusion protein between the 181 N-terminal amino acids of β -lactamase¹⁰ and L. The detection of both polypeptides was dependent on transcription from *P_L*; in repressed conditions neither was observed (Fig. 1b, lane 1), nor if the orientation of the genes was reversed with respect to *P_L* (data not shown). *Synechococcus* S could not be identified in Fig. 1b, lane 2, when using anti-wheat RuBPCase serum because of the greater evolutionary divergence of S compared with L polypeptides. To demonstrate S synthesis, derepressed *E. coli* cells containing pSV55 were labelled with ³⁵S-methionine and the assembled soluble enzyme complex was separated from unassembled subunits by gel filtration, then reacted with anti-wheat RuBPCase. The immune complex was adsorbed to protein A-Sepharose, thus trapping assembled S via binding to L, which in turn interacts with IgG and protein A. Figure 1, lane 4, shows that both L and S are present together in a subunit complex excluded from a Biogel P-300 column (exclusion limit 400,000 M_r).

It has been shown that the presence of both S and L are essential for the catalytic function of *Synechococcus* RuBPCase¹¹. The soluble fractions of sonicated *E. coli* cells containing pSV55, with or without induction, were prepared and used to measure RuBPCase activity. Figure 2a shows the results of assaying for RuBP-dependent incorporation of ¹⁴CO₂ into acid-stable products. Induced cell extracts show a linearity of ¹⁴C incorporation with time and a specific activity of 8 nmol CO₂ fixed per min per mg soluble protein at 30 °C. In contrast, the uninduced cell extract contains no detectable RuBPCase activity. The RuBPCase activity present in induced cell extracts can be inhibited by the addition of anti-wheat RuBPCase serum before assay (Fig. 2b). To confirm that RuBPCase activity was dependent on the presence of S, the following deletion experiment was devised. The 2.17-kb *Pst*I fragment was transferred from pSV55 to the *Pst*I site of pUC9 (ref. 12) in the appropriate orientation for the L and S genes to be transcribed from the *lac* promoter. The resulting plasmid, pDB50, when induced with isopropyl β -D-thiogalactoside, gave a specific activity for RuBPCase of 14 nmol CO₂ fixed per min per mg soluble protein at 30 °C when assayed as described in Fig. 2 legend. Plasmid

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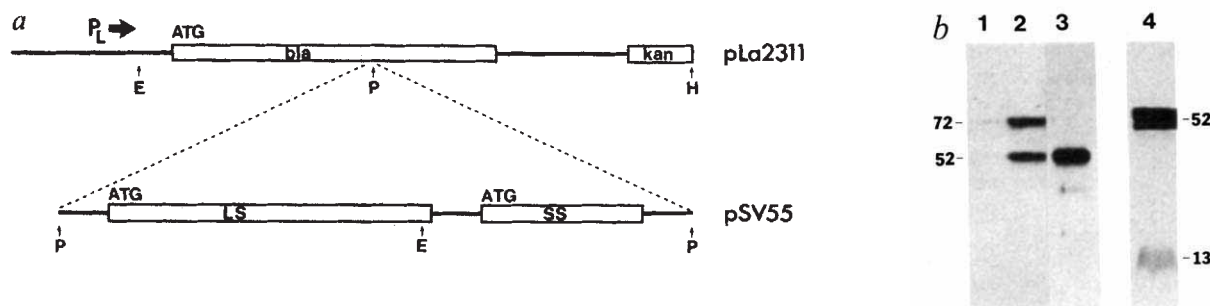


Fig. 1 *a*, Construction of RuBPCase expression plasmids. There are asymmetrical *EcoRI* sites in the vector and insert. The plasmid in which the insert is orientated for RuBPCase gene transcription from P_L is designated pSV55. The position of the *PstI* (P), *EcoRI* (E) and *HindIII* (H) sites are indicated, together with the large subunit (LS), small subunit (SS) and β -lactamase (*bla*) genes with their initiator methionine (ATG) marked. The direction of transcription from the P_L promoter is shown with an arrow. *b*, Analysis of RuBPCase polypeptide synthesis in M5219 cells containing pSV55 plasmid. Lane 1, total protein of uninduced pSV55 (Western blot); lane 2, total protein of induced pSV55 (Western blot); lane 3, total maize leaf protein (Western blot); lane 4, immunoadsorbed pSV55-encoded L and S polypeptides labelled with ^{35}S *in vivo*.

Methods. *a*, The *Synechococcus* RuBPCase L and S genes are present on a 2.17-kb *PstI* fragment in the clone pLS506 (ref. 8). The pLS506 plasmid was digested with *PstI* and ligated into the P_L expression plasmid pLa2311 (ref. 9), previously linearized with *PstI* at a site within the ampicillin-resistance gene. The kanamycin-selectable marker on pLa2311 was retained intact. The ligation mixture was transformed into strain M5219 [M72*lacZ*amtrpamSm^R(λ bio252cI857 Δ H1)] (ref. 9) and kanamycin-resistant colonies selected. Ampicillin-sensitive colonies containing the 2.17-kb *PstI* insert were digested with *EcoRI* to identify clones with the two possible orientations of the fragment. *b*, To detect LS synthesis, M5219 cells containing pSV55 were grown at 30 °C in 100 ml of L broth containing 50 $\mu\text{g ml}^{-1}$ kanamycin to $A_{500} = 0.4$. Cells were induced at 45 °C for 10 min and then cultured at 41 °C for 2 h with vigorous shaking. Uninduced controls were maintained at 30 °C. Cells were collected by centrifugation and incubated for 30 min on ice in 0.5 ml of 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mg ml⁻¹ lysozyme. The solution was sonicated for 30 s, mixed with 0.5 ml 10% (w/v) SDS and incubated at 25 °C for 10 min. A soluble fraction was recovered after centrifugation in an Eppendorf microfuge for 15 min. Maize leaf proteins were isolated by grinding 1 g of young tissue with 3 ml of 100 mM Tris-HCl pH 8.0, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM EDTA, 1 mM β -mercaptoethanol and 2% (w/v) insoluble polyvinylpyrrolidone (PVP; buffer A). The homogenate was clarified using an Eppendorf microfuge for 5 min. Soluble proteins from bacterial and leaf cells were heated in SDS loading buffer and electrophoresed on a 15% polyacrylamide gel containing SDS¹⁹. Polypeptides were transferred from the gel to nitrocellulose²⁰, the filters preincubated with 1.5% (w/v) gelatin in 10 mM Tris-HCl pH 7.5, 0.35 M NaCl, for 2 h, and then incubated with 25 μl anti-wheat RuBPCase serum overnight in 25 ml 10 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.1% deoxycholate, 0.1% SDS and 1% Triton X-100 (RIA buffer). After washing with RIA buffer, the filter was incubated with 0.5 μCi ¹²⁵I-protein A for 2 h and then washed in RIA buffer before autoradiography. To detect SS synthesis, a 25-ml culture of K-12 Δ H1 Δ trp cells⁹ containing pSV55 was grown in M9 medium containing glucose, tryptophan, biotin and thiamine-HCl to $A_{600} = 0.5$ at 30 °C. The culture was shifted to 42 °C and after 5 min 2 mCi ³⁵S-methionine were added. After 1.5 h at 42 °C, the cells were collected by centrifugation, resuspended in 0.5 ml 25% (w/v) sucrose, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mg ml⁻¹ lysozyme for 15 min at 0 °C, and then lysed by the addition of an equal volume of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM EDTA, 1 mM β -mercaptoethanol and 0.1% (v/v) Triton X-100 (buffer B). Phenylmethylsulphonyl fluoride was added to 1 mM, the lysate sheared through a hypodermic syringe and then centrifuged at 100,000g for 1 h. The supernatant (1 ml) was passed through a 0.9 $\times$ 27 cm column of Biogel P-300 (50–100 mesh) in buffer B at 4 °C. The excluded column fractions ($M_r \geq 400,000$) were incubated with anti-wheat RuBPCase and then adsorbed to protein A-Sepharose and washed as before^{21,22} in non-denaturing conditions. The adsorbed polypeptides were boiled in SDS sample buffer, resolved by electrophoresis on a 20% polyacrylamide gel containing SDS¹⁹ and fluorographed²³. Gels were calibrated with ¹⁴C-labelled markers as before^{21,22}.

pDB50 was digested with *PstI* and partially with *HindIII* (see ref. 8) and a resulting 1.7-kb fragment containing only the L gene transferred to the *PstI*/*HindIII* sites of pUC8 (ref. 12), such that the L gene was transcribed from the *lac* promoter. Induction of the resulting plasmid pDB53 did not yield any detectable RuBPCase activity, indicating that S is required for the active enzyme complex to form in *E. coli*. Western blotting confirmed that L synthesis was still directed by pDB53 (data not shown).

For further probing of the active site, the transition-state analogue 2-carboxyarabinitol 1,5-bisphosphate (CABP) was used. This binds to the activated ternary complex $\text{E} \cdot \text{CO}_2 \cdot \text{Me}^{2+}$ to give a stable quaternary complex of $\text{E} \cdot \text{CO}_2 \cdot \text{Me}^{2+} \cdot \text{CABP}$ (ref. 1). A mixture of ¹⁴C-labelled diastereomers CABP and 2-carboxyribitol 1,5-bisphosphate (CRBP) were synthesized¹³ and then incubated with soluble extracts of control *E. coli* cells, induced cells containing pSV55, or purified spinach RuBPCase. The extracts were passed through affinity columns containing immobilized anti-wheat RuBPCase and the immunoadsorbed protein was eluted with 8 M urea¹⁴. Column fractions were counted for ¹⁴C isotope content and aliquots analysed by Western blotting. In Fig. 3a, labelled peaks can be seen for the spinach RuBPCase and the pSV55 extract, but not for the control strain. The labelled analogue binding, together with the antibody column, has therefore allowed the detection of specific binding to the active site of the *Synechococcus* enzyme synthesized in *E. coli*. Figure 3b confirms that the protein adsorbed to the column contains the L polypeptide.

RuBPCase isolated from *Synechococcus* has a M_r of 530,000

(ref. 6) with subunit M_r s of 52,470 for L^S and 14,000 for S¹⁵, and a hexadecameric structure of L₈S₈. To analyse the structural complexity of the enzyme synthesized in *E. coli*, soluble protein extracts from induced cells containing pSV55 were centrifuged on sucrose gradients. The position of the enzyme in the gradient was determined either by RuBPCase activity or by prior labelling of the active site with ¹⁴C-labelled CABP/CRBP. The protein samples layered on the gradients contained β -galactosidase as an internal marker, and gradients centrifuged in parallel contained spinach RuBPCase (M_r 550,000), β -galactosidase (465,000), aldolase (160,000) and ovalbumin (43,000). Figure 4a, b shows that the size of RuBPCase synthesized in *E. coli* is similar to spinach RuBPCase. Sucrose gradient fractions analysed by SDS-gel electrophoresis and Western blotting using anti-wheat RuBPCase serum revealed the presence of the *Synechococcus* L polypeptide in the fractions containing the RuBPCase peak of activity (Fig. 4c). There was also some β -lactamase/L fusion protein in the lower part of the gradient, although most of this 72,000- M_r protein is located in the membrane fraction, presumably because of initiation of export by the β -lactamase signal sequence¹⁶. It does not necessarily follow that a hexadecameric structure is formed in *E. coli*, because the RuBPCase synthesized in the bacteria and spinach leaves is of a similar size, as determined by sucrose gradient centrifugation and by electrophoresis on non-denaturing gels (data not shown). The large size indicates that the L₈ structure is probably formed, but an L₈S_n complex where n is less than 8 would be difficult to resolve because of the small size of S. Although S must be present to obtain an active enzyme¹⁵, we believe that the S

binding sites on the L octamer may not be fully saturated because of two observations. First, we can estimate from ^{14}C -labelled CABP/CRBP binding to L_8S_n complexes on sucrose gradients that these structures comprise about 2% of the *E. coli* soluble

proteins and yet the specific activity of RuBPCase in crude extracts is lower than expected. Second, liquid scintillation counting of ^{35}S present in the L and S polypeptides isolated from SDS-polyacrylamide gels (Fig. 1b, lane 4) indicates that L_8S_n structures are formed where $n=2$ or 3. This problem is likely to be resolved by increased production of S relative to L using suitable plasmids.

Previous work has shown that active RuBPCase can be synthesized in *E. coli* using genes from purple non-sulphur bacteria to give either a dimeric L_2 (ref. 17) or a hexameric L_6 (ref. 18) enzyme. These enzymes, however, are atypical in that an S polypeptide is not required for activity. The most common form of the enzyme is the L_8S_8 structure found in plants, and in prokaryotes possessing a Calvin cycle. In the L_8S_8 structures, the presence of S is essential for activity, based on evidence from *Synechococcus*¹⁵ and from our data presented here in which deletion of the S gene abolishes RuBPCase activity. A suggested role for S is that its binding to L induces a conformational change that promotes activity, and so it has an essential but indirect role in catalysis¹⁵. This report, describing the assembly in *E. coli* of an L_8 structure containing S subunits to enable the enzyme to fix CO_2 , represents one of the most complex macromolecular structures to be synthesized using recombinant DNA techniques. The RuBPCase activity is presumably

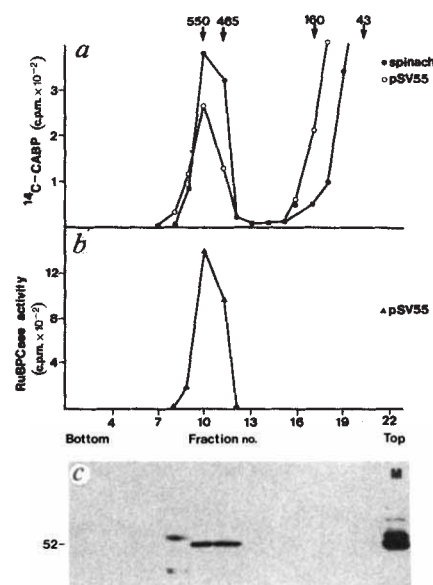
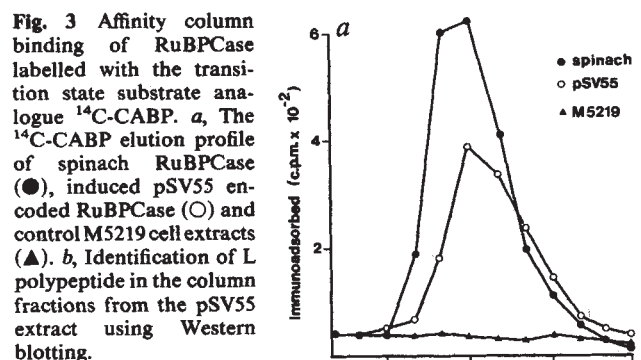
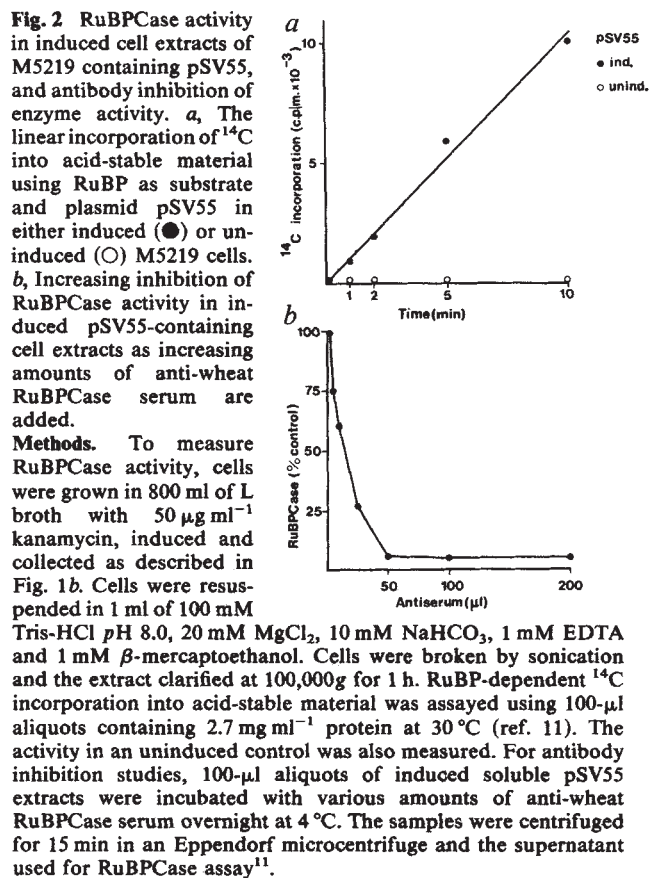


Fig. 4 Velocity sedimentation of RuBPCase in sucrose gradients. **a**, Gradient profile of ^{14}C -CABP-treated protein using spinach RuBPCase (●) or induced pSV55-containing cells (○). **b**, Distribution of RuBPCase activity on a sucrose gradient using induced pSV55-containing cells (▲). **c**, Western blot of the gradient fractions shown in panel b. The M track is a maize leaf extract used as a marker. M, markers shown by arrows.

Methods. Soluble protein extracts were prepared as described in Fig. 2 from induced and uninduced M5219 cells containing pSV55. β -Galactosidase was added as a marker to these extracts and 500 μl was layered on to an 11-ml linear 7–25% sucrose gradient in 0.1 M Tris-HCl pH 8.0, 20 mM MgCl_2 , 10 mM NaHCO_3 , 1 mM EDTA, 1 mM mercaptoethanol. The gradients were centrifuged for 24 h at 25,000 r.p.m. and 4°C in a Sorvall TST41 swinging bucket rotor. Fractions (0.5 ml) were collected from the bottom of the tube and assayed for RuBPCase activity¹¹. Alternatively, the protein extract was incubated with ^{14}C -CABP at $10\ \mu\text{M}$ concentration for 1 h before centrifuging. In this case, the gradient fractions were counted directly for ^{14}C content. Aliquots of fractions were also analysed by electrophoresis and Western blotting to locate the position of the L polypeptide on the gradient. All gradients had an internal marker, β -galactosidase (M_r 465,000), that could be assayed directly²⁴. In addition, identical calibration gradients were centrifuged at the same time containing spinach RuBPCase (M_r 550,000), β -galactosidase (465,000), aldolase (160,000) and ovalbumin (43,000).

expressed by LS pairs present in the L_nS_n structures irrespective of the value of n . This system should provide an opportunity to gain further understanding of the assembly mechanisms and the importance of subunit contact sites in forming and maintaining the structure of the holoenzyme.

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Note added in proof: By increasing the expression of S, we find that RuBPCase activity is also enhanced, suggesting that the S binding sites on the L octamer can be increasingly saturated by S overproduction.

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1. Mizioro, H. M. & Lorimer, G. H. *Rev. Biochem.* **52**, 507-535 (1983).
2. Ellis, R. J. & Gatenby, A. A. *The Genetic Manipulation of Plants and its Application to Agriculture* (eds Lea, P. J. & Stewart, G. R.) 41-60 (Oxford University Press, 1984).
3. Gatenby, A. A. *Eur. J. Biochem.* **144**, 361-366 (1984).
4. Voordouw, G., van der Vies, S. M. & Bouwmeester, P. P. *Eur. J. Biochem.* **141**, 313-318 (1984).
5. Shinozaki, K., Yamada, C., Takahata, N. & Sugiura, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4050-4054 (1983).
6. Andrews, T. J., Abel, K. M., Menzel, D. & Badger, M. R. *Archs Microbiol.* **130**, 344-348 (1982).
7. Shinozaki, K. & Sugiura, M. *Nucleic Acids Res.* **11**, 6957-6964 (1983).
8. Reichelt, B. Y. & Delaney, S. F. *DNA* **2**, 121-129 (1983).
9. Remaut, E., Stanssens, P. & Fiers, W. *Gene* **15**, 81-93 (1981).
10. Sutcliffe, J. G. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3737-3741 (1978).
11. Andrews, T. J. & Abel, K. M. *J. biol. Chem.* **256**, 8445-8451 (1981).
12. Vieira, J. & Messing, J. *Gene* **19**, 259-268 (1982).
13. Rabin, B. R., Shaw, D. F., Pon, N. G., Anderson, J. M. & Calvin, M. J. *Am. chem. Soc.* **80**, 2528-2532 (1958).
14. Gatenby, A. A. & Cocking, E. C. *Pl. Sci. Lett.* **10**, 97-101 (1977).
15. Andrews, T. J. & Ballment, B. J. *biol. Chem.* **258**, 7514-7518 (1983).
16. Kadonaga, J. T. *et al. J. biol. Chem.* **259**, 2149-2154 (1984).
17. Somerville, C. R. & Somerville, S. C. *Molec. gen. Genet.* **193**, 214-219 (1984).
18. Quivey, R. G. & Tabita, F. R. *Gene* **31**, 91-101 (1984).
19. Laemmli, U. K. *Nature* **227**, 680-685 (1970).
20. Vaessen, R. T. M. J., Kreike, J. & Groot, G. S. P. *FEBS Lett.* **124**, 193-196 (1981).
21. Gatenby, A. A., Castleton, J. A. & Saul, M. W. *Nature* **291**, 117-121 (1981).
22. Gatenby, A. A. & Castleton, J. A. *Molec. gen. Genet.* **185**, 424-429 (1982).
23. Bonner, W. M. & Laskey, R. A. *Eur. J. Biochem.* **46**, 83-88 (1974).
24. Miller, J. H. *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, New York, 1972).

Renal cytochrome P450-related arachidonate metabolite inhibits $(Na^+ + K^+)ATPase$

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The function of the nephron, the anatomical unit of the kidney, is segmented; at least 12 segments have been identified that differ in their morphology, transport properties and hormonal responsiveness¹. The medullary portion of the thick ascending limb of the loop of Henle (mTALH) has one of the highest concentrations of $(Na^+ + K^+)ATPase$ found in mammalian tissues², reflecting the importance of this nephron segment in the regulation of extracellular fluid volume, as active sodium transport is driven by $(Na^+ + K^+)ATPase$ ¹. Here, in cells derived primarily from the mTALH of the outer medulla of rabbit kidney, we have identified a cytochrome P450-dependent monooxygenase system³ which metabolizes arachidonic acid to two biologically active oxygenated products; one of the products inhibits $(Na^+ + K^+)ATPase$ and the other relaxes blood vessels. We report that formation of these oxygenated arachidonate metabolites is stimulated by arginine vasopressin (AVP) and salmon calcitonin (SCT).

Male New Zealand White rabbits (3.0-3.5 kg) were anaesthetized with sodium pentobarbital (30 mg per kg), and the inner stripe of the outer medulla excised, cut into small pieces and trypsinized for 20 min in trypsin-EDTA (1×) plus 0.1% bovine

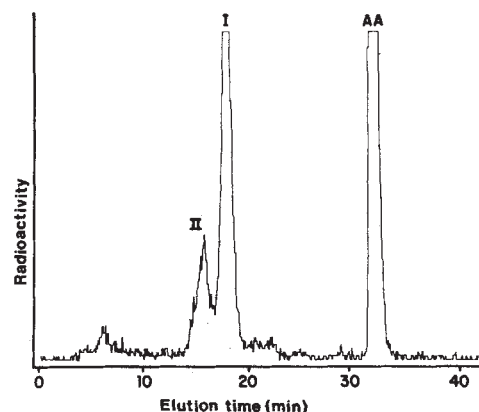


Fig. 1 Reverse-phase chromatography of arachidonic acid metabolites formed by mTALH cells. The retention times of peaks I and II were 18.2 and 15.7 min, respectively, while those for the monohydroxyicosatetraenoic acids (mono-HETEs) in this HPLC separation were as follows: 15-HETE, 19 min; 11-HETE, 20 min; 12-HETE, 21 min; 9-HETE, 22 min; 5(S),12(S)-diHETE, 17.5 min.

Methods. Cells ($3 \times 10^6 \text{ ml}^{-1}$) were incubated in phosphate-buffered saline (PBS) with $0.4 \mu\text{Ci } ^{14}\text{C}$ -arachidonic acid ($7 \mu\text{M}$; specific activity 56 mCi mmol^{-1}) at 37°C for 30 min. The reaction was stopped by acidification to pH 4.0, the cells were removed by centrifugation and the media extracted with ethyl acetate. Arachidonic acid metabolites were separated by HPLC on a μ Bondapak C_{18} column, using a linear solvent gradient ranging from water:acetonitrile (1:1, containing 0.1% acetic acid) to 100% acetonitrile (containing 0.1% acetic acid); the rate of change was 1.25% per min at a flow rate of 1 ml min^{-1} . Radioactive products were detected using a radioactive flow detector. Fractions containing peaks I and II were collected separately, methylated with a freshly prepared solution of diazomethane in ether and subjected to further purification on normal-phase HPLC using a μ Porasil column and solvent system comprising hexane/isopropanol/acetic acid (98:2:0.1) at a flow rate of 1 ml min^{-1} . The purified methylated products were further derivatized to the trimethylsilyl derivatives and subjected to GC-MS analysis, using a Hewlett-Packard HP-5985A GC-MS system. The samples were separated on a glass column (1% SE-30). Operational conditions of the GC-MS were: injection port 250°C ; initial temperature of 180°C increasing at a rate of 8°C min^{-1} up to 250°C ; interface 275°C ; ion source 300°C and electron impact energy of 70 eV. GC-MS analysis revealed that each peak separated by HPLC contained more than one compound. A major compound associated with peak II can be defined as the methyl ester-trimethylsilyl derivative of 11,12-dihydroxyicosatrienoic acid because the fragments at $m/z = 496 (M^+)$, $465 (M^+ - 31)$, $406 (M^+ - 90)$, $385 (M^+ - 111)$, $315 (M^+ - 181)$, 295 and 213 are characteristic of this structure. A major compound associated with peak I has prominent fragments at $m/z = 316 (M^+ - 18)$, $303 (M^+ - 31)$, 233, 190 and 176, suggesting the presence of the methyl ester derivative of 5,6-epoxyicosatrienoic acid.

serum albumin. A single-cell suspension of outer medullary cells was prepared and separated into several fractions by centrifugal elutriation^{3,4}. Cells derived mainly from the mTALH were eluted at a flow rate of 19 ml min^{-1} at 2,000 r.p.m., at 20°C . Over 90% of the cells were viable as determined by Trypan blue exclusion, electron microscopic appearance and metabolic activity. After cell separation, ~80% of the cells proved to be mTALH in origin, based on electron microscopic criteria and immunofluorescent localization of Tamm-Horsfall protein, a specific marker for mTALH cells⁵. The remainder were cells derived from proximal tubules and collecting ducts. The specific activity of alkaline phosphatase, a marker for proximal tubule cells⁶, decreased threefold after separation of mTALH cells from outer medullary cells, in association with a fourfold increase in the ability of the separated mTALH cells to metabolize arachidonic acid to oxygenated metabolites.

Incubation of mTALH cells with ^{14}C -labelled arachidonic acid resulted in formation of oxygenated metabolites, identified as peaks I and II (see Fig. 1), which accounted for 30-40% of the recovered radioactivity. Formation of the arachidonate

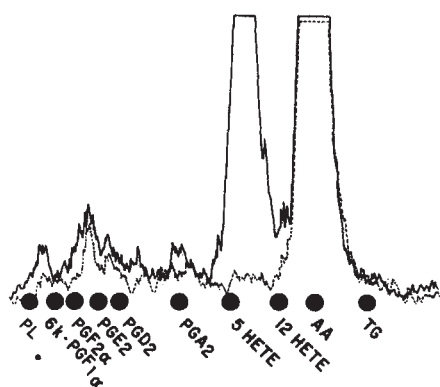


Fig. 2 Arachidonic acid metabolism by a broken cell preparation of mTALH cells. Cells were lysed by two cycles of freeze-thaw with liquid nitrogen in distilled water, then adjusted to PBS, pH 7.4. Broken cell preparations (200 μ l, 80–100 μ g protein) were incubated with 14 C-arachidonic acid (0.2 μ Ci) plus 'cold' arachidonic acid (10 μ M) in the presence (—) and absence (---) of NADPH (1 mM) at 37 °C for 30 min. The reaction was stopped by acidification to pH 4.0 and extracted with ethyl acetate. The final extract was subjected to TLC for separation of arachidonic acid oxygenated metabolites using the upper phase of ethyl acetate/isooctane/acetic acid/water (110:50:20:100). PL, phospholipids; 6 k-PGF $_{1\alpha}$, 6-keto-prostaglandin F $_{1\alpha}$; PGE $_2$, PGD $_2$ and PGA $_2$, prostaglandin E $_2$, D $_2$ and A $_2$, respectively; TG, triglycerides; HETE, hydroxyeicosatetraenoic acid; AA, arachidonic acid. The radioactive peak appearing between the 5- and 12-HETE standards consists of peak I and peak II defined in Fig. 1. Formation of prostaglandins accounted for only 3–5% of the radioactivity recovered.

metabolites constituting peaks I and II was not affected by indomethacin⁷ (3 μ M), while eicosatetraenoic acid (30 μ M), the acetylenic analogue of arachidonate⁸, inhibited product formation by 80–90%. Separation by reverse-phase HPLC revealed that the arachidonate metabolites of peaks I and II had retention times different from those of arachidonate products of lipoxygenases, the mono- and dihydroxyeicosatetraenoic acids. Further, the lack of absorbance at 234 nm and 276 nm indicated the absence of a conjugated diene or triene structure, respectively, and suggested that the oxygenated metabolites of arachidonic acid were formed by a cytochrome P450-dependent system. At 50 μ M, SKF-525A (β -diethylaminoethyl-diphenylpropyl acetate hydrochloride), an inhibitor of cytochrome P450-dependent enzymes⁹, reduced product formation by mTALH cells by more than 70% (from 2.52 ± 0.66 μ g to 0.27 ± 0.06 μ g of arachidonic acid converted per mg protein per 30 min), while induction of cytochrome P450 with 3-methylcholanthrene and β -naphthoflavone¹⁰ (40 mg per kg per day on each of 3 days, injected intraperitoneally) increased arachidonic acid conversion to 4.17 ± 1.02 μ g per mg protein per 30 min. In addition, product formation by cell-free homogenates of mTALH was dependent on the presence of NADPH (Fig. 2), suggesting that arachidonic acid metabolism in mTALH cells occurs via an NADPH-dependent cytochrome P450-linked monooxygenase pathway¹¹. In view of studies based on immunofluorescent¹² and microanalytical¹³ methods that have assigned the cytochrome P450-monooxygenase system to the proximal tubules, the present study, which has localized this pathway of arachidonate metabolism also in the mTALH, should be interpreted with caution.

Segmentation of nephron responsiveness to hormones may be expressed through adenylate cyclase-sensitive receptors¹⁴. AVP-dependent adenylate cyclase activity is restricted to the collecting ducts and mTALH¹⁵. Calcitonin also stimulates adenylate cyclase in the mTALH whereas adrenocorticotrophic hormone, parathyroid hormone and isoprenaline do not¹⁴. AVP and SCT, when added to mTALH cells in concentrations ranging from 10^{-10} – 10^{-7} M, stimulated release of the oxygenated

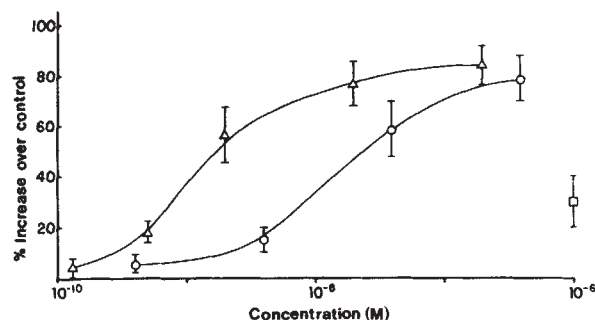


Fig. 3 Hormonal stimulation of arachidonic acid metabolism in mTALH cells prelabelled with 14 C-arachidonic acid (0.4 μ Ci per 3×10^6 cells) for 90 min at 37 °C. Within 90 min, 80–90% of 14 C-arachidonic acid was incorporated into phospholipids, chiefly phosphatidylcholine and phosphatidylethanolamine, and triglycerides. The cell suspension was centrifuged, then the cells were resuspended in fresh PBS and incubated for an additional 10 min with the following peptide hormones: AVP (O, 4×10^{-10} – 4×10^{-7} M), SCT (Δ , 10^{-10} – 2×10^{-7} M) and bradykinin ($\square$, 10^{-6} M). The reaction was stopped by acidification, cells were removed by centrifugation and the media extracted with ethyl acetate. Arachidonic acid metabolites were separated by TLC as described in Fig. 2 legend; radioactive zones corresponding to peaks I and II were cut out and counted using a liquid scintillation counter. The per cent increase in the formation of peaks I and II compared with the unstimulated control value was plotted. The control value of 0.060 ± 0.007 μ g per mg protein per 10 min is considerably lower than the values obtained when the 90-min period of prelabelling was omitted, as it represents release of bound radiolabelled arachidonic acid which was esterified to lipids during prelabelling. Values are mean $\pm$ s.e.m. of four experiments.

arachidonate metabolites of peaks I and II by as much as twofold, depending on the dose of the peptides (Fig. 3). In contrast, concentrations of bradykinin and angiotensin II that were three orders of magnitude greater, had lesser effects on the release of oxygenated metabolites of arachidonic acid than either AVP or SCT. The phosphodiesterase inhibitor, 1-isobutyl-3-methylxanthine (10 μ M), increased formation of peaks I and II almost twofold, as did dibutyryl cyclic AMP (1 mM). Parathyroid hormone and isoprenaline, which do not affect adenylate cyclase in the mTALH segment¹², had no effect on arachidonic acid metabolite formation by mTALH cells at concentrations of 10^{-10} – 10^{-7} M.

Peak II proved to be a potent inhibitor of ($\text{Na}^+ + \text{K}^+$)ATPase whereas peak I was a weak inhibitor (Table 1). Peak I but not peak II relaxed rabbit pulmonary artery rings that had been precontracted with phenylephrine (5×10^{-7} M); peak I (4×10^{-8} – 2.3×10^{-7} M, $n=4$) resulted in a 40–60% relaxation whereas higher doses of peak II (2.9×10^{-7} – 1.2×10^{-6} M, $n=3$) had no detectable effects. Acetylcholine, used as a standard, caused a 36% relaxation of vascular rings at a dose of 5×10^{-8} M. Gas chromatography-mass spectrometry (GC-MS) analysis of peaks I and II revealed major components consistent with the structures of the 5,6-epoxide and the 11,12-diol derivatives of arachidonic acid, respectively (see Fig. 1 legend).

The results of our study indicate that renomedullary cells respond selectively to hormonal stimuli by generating arachidonic acid metabolites via a cytochrome P450-dependent mechanism, and that one of these products inhibits ($\text{Na}^+ + \text{K}^+$)ATPase. A hormone-inducible modulator of ($\text{Na}^+ + \text{K}^+$)ATPase activity has long been sought¹⁶ and has important consequences for the regulation of extracellular fluid volume and composition. These findings also have a bearing on selective activation of the nephron by hormones via stimulation of adenylate cyclase-sensitive segments such as those of the cortical collecting ducts and mTALH by AVP¹⁴. Moreover, the concentrations of AVP¹⁵ and SCT¹⁴ that stimulate adenylate cyclase activity in mTALH correspond to concentrations of these peptides which we have shown to increase formation of arachidonic

acid metabolites in this segment, that is, 10^{-10} – 10^{-8} M. As the effects of SCT and AVP on arachidonic acid metabolism are mimicked by dibutyryl cyclic AMP, the natriuretic effects of SCT¹⁷ and AVP¹⁸ may be mediated by adenylate cyclase-induced arachidonic acid metabolism. A natriuretic effect of AVP which is localized to mTALH¹⁸ has been dissociated from its effect on reabsorption of water¹⁹. Finally, a factor which diminishes the activity of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ in the mTALH has been postulated by Postnov *et al.*²⁰ to account for the exaggerated natriuresis in response to volume expansion in clinical and experimental hypertension. In this regard, we have demonstrated more than a twofold increase in formation of cytochrome P450-related products of arachidonic acid metabolism by TALH cells in rabbits made hypertensive by constriction of the abdominal aorta²¹. In conclusion, the cytochrome P450-dependent pathway of arachidonate metabolism in mTALH could act as a common mechanism mediating natriuresis in response to hormones as well as to a natriuretic substance(s)²² including circulating inhibitors of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activity, levels of which are thought to be elevated in hypertension²³.

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Table 1 Effect on $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ of arachidonic acid metabolites formed by renomedullary cells, primarily from the thick ascending limb of rabbit kidney

	Concentration* (μM)	% Inhibition of $(\text{Na}^+ + \text{K}^+)\text{ATPase}^\dagger$
Peak I	0.05	No effect
	0.28	10
	2.60	35
Peak II	0.06	34
	0.20	65
	0.66	70

ATPase inhibition was assayed using a microsomal preparation from canine heart, prepared and treated with 6 M sodium iodide, followed by 1.5 M deoxycholate-citrate. The final pellet was dialysed overnight at 4 °C against imidazole-EDTA (25 mM–1 mM) and stored at –50 °C according to the method of Pitts and Schwartz²⁴. This enzyme was 98% ouabain (1 mM)-inhibitable, with an activity of 231 μmol inorganic phosphate per mg protein per h. The 50% inhibitory concentration of ouabain for this enzyme preparation was 0.5 μM . The effects of peaks I and II on $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activity, in the presence and absence of 1 mM ouabain, were measured according to the method of Martin and Doty²⁵. Inorganic phosphate was measured spectrometrically by the direct addition of stannous chloride solution to an aliquot of the extract which had been diluted with ethyl alcohol-sulphuric acid solution. For the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ assay, 15 μg of enzyme was incubated for 30 min at 37 °C in 1 ml of a reaction mixture containing 50 mM Tris buffer, 5 mM MgCl_2 , 100 mM NaCl, 10 mM KCl and 0.5 mM EDTA. The reaction was started by adding ATP (5 mM) and stopped by adding 10% trichloroacetic acid. Peaks I and II (Fig. 1) are the pooled fractions (separated by reverse-phase HPLC) of oxygenated arachidonic acid metabolites formed by mTALH cells obtained from three rabbits. An identical volume of the HPLC effluent similarly extracted did not inhibit $(\text{Na}^+ + \text{K}^+)\text{ATPase}$.

* The concentrations of peaks I and II were calculated from per cent conversion of ¹⁴C-arachidonic acid (after correction for extraction efficiency) to these metabolites assuming a molar ratio of 1:1 for each; that is, mole of arachidonic acid converts to either mole of peak I or peak II. The concentrations were estimated on the basis of a relative molecular mass of 350 for oxygenated metabolites of arachidonic acid. The estimated amount of arachidonic acid products (peaks I and II) produced by 3×10^6 cells ml^{-1} was ~400 ng in 30 min of incubation. Thus, the approximate concentration of the arachidonate metabolite(s) in the incubate was 0.1 μM , a biologically effective concentration as judged by the inhibition of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$.

† Inhibition of ouabain-sensitive $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. Each value is the mean of duplicate determinations of $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ inhibitory activity of arachidonic acid products contained in peaks I and II. Student's paired *t*-test indicated a significant difference between the activity in the two peaks (*t* = 6.03, d.f. = 2, *P* < 0.05).

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- Knepper, M. & Burg, M. *Am. J. Physiol.* **244**, F579–F589 (1983).
- Jørgensen, P. L. *Physiol. Rev.* **60**, 864–917 (1980).
- Ferreri, N. R., Schwartzman, M., Ibrahim, N. G., Chander, P. N. & McGiff, J. C. *J. Pharmac. exp. Ther.* **231**, 441–448 (1984).
- Grabske, R. J., Lake, S., Gledhill, B. L. & Meistrich, M. L. *J. cell. Physiol.* **86**, 177–190 (1975).
- Floyer, J. R., Sisson, S. P. & Vernier, R. L. *Lab. Invest.* **41**, 168–173 (1979).
- Copenhaver, W. M., Kelly, D. E. & Wood, R. L. in *Bailey's Textbook of Histology* (eds Copenhaver, W. M., Kelly, D. E. & Wood, R. L.) 585–587 (Williams & Wilkins, Baltimore, Maryland, 1978).
- Vane, J. R. *Nature new Biol.* **231**, 232–235 (1971).
- Downing, D. T., Ahern, D. G. & Bacht, M. *Biochem. biophys. Res. Commun.* **40**, 218–223 (1970).
- Testa, B. & Jenner, P. *Drug Metab. Rev.* **12**, 1–117 (1981).
- Zenser, T. V., Mattammal, M. B. & Davis, B. B. *J. Pharmac. exp. Ther.* **207**, 719–725 (1978).
- Capdevila, J., Marnett, L. J., Chacos, N. N., Prough, R. A. & Estabrook, R. W. *Proc. natn. Acad. Sci. U.S.A.* **79**, 767–770 (1982).
- Endou, H. *Jap. J. Pharmac.* **33**, 423–433 (1983).
- Dees, J. H., Masters, B. S. S., Muller-Eberhard, U. & Johnson, E. F. *Cancer Res.* **42**, 1423–1432 (1982).
- Morel, F. *Am. J. Physiol.* **240**, F159–F164 (1981).
- Imbert-Teboul, M., Chabardes, D., Montégut, M., Clique, A. & Morel, F. *Endocrinology* **102**, 1254–1261 (1978).
- Katz, A. I. *Am. J. Physiol.* **242**, F207–F219 (1982).
- Williams, C. C., Matthews, E. W., Moseley, J. M. & MacIntyre, I. *Clin. Sci.* **42**, 129–137 (1972).
- Fejes-Tóth, G. & Szénási, G. *J. Physiol., Lond.* **318**, 1–7 (1981).
- Morel, F. *Bull. biol. Fr. Belg. Suppl.* **39**, 136 (1955).
- Postnov, Y. U., Reznikova, M. & Boriskina, G. *Pflügers Arch. ges. Physiol.* **362**, 95–99 (1976).
- Carrara, M. C., Carroll, M. A., McGiff, J. C. & Schwartzman, M. *Br. J. Pharmac. Abstr.* (in the press).
- deWardener, H. E. *et al. Lancet* **i**, 411–412 (1981).
- Overbeck, H. W., Pamnani, M. B., Akera, T., Brody, T. M. & Haddy, F. J. *Circulation Res.* **38**, 11–48–11–52 (1976).
- Pitts, B. J. R. & Schwartz, A. *Biochim. biophys. Acta* **401**, 184–195 (1975).
- Martin, J. B. & Doty, D. M. *Analyt. Chem.* **21**, 965–967 (1949).

Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly *in vivo*

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The recognition that the endoplasmic reticulum (ER), rather than the mitochondria, is the main organelle regulating the cytoplasmic Ca^{2+} concentration in non-muscle cells^{1,2} supports the notion that an alternative physiological role of mitochondrial Ca transport^{3–5} is the modulation of Ca -sensitive mitochondrial enzymes through small (micromolar) fluctuations in the concentration of mitochondrial matrix Ca^{2+} (refs 1, 5–7). The latter mechanism could operate only if the mitochondrial Ca concentration were low, as it is in muscle and retinal rods^{8,9}, below the levels saturating the regulated enzymes⁵. In contrast, if the ER serves as an intracellular Ca store, its Ca content would be expected to be high. In view of the major metabolic function of the liver, the question of whether hepatic mitochondrial matrix Ca^{2+} regulates metabolism is particularly important, but the range of Ca concentrations reported for isolated liver mitochondria is too wide^{10–12} to provide a conclusive answer. Therefore, we have used electron probe X-ray microanalysis (EPMA) to measure the subcellular distribution of Ca in liver snap-frozen *in vivo*, and report here that the endoplasmic reticulum is a major intracellular store of Ca , while the concentration of Ca in mitochondria is low and compatible with the regulation of mitochondrial enzymes.

Figure 1 shows an electron micrograph of an unstained cryo-section of rat liver used for electron probe analysis; there is minimal ice-crystal damage, allowing the identification of cell types and intracellular organelles. The Ca concentration in red blood cells within hepatic sinusoids in the same sections used for parenchymal cell measurements, 0.1 ± 0.2 mmol per kg dry wt ($\pm$ s.e.m.; *n* = 34), is consistent with the micromolar total Ca content of red blood cells¹³ and provides an internal estimate

Fig. 1 Electron micrograph of an ultrathin, freeze-dried cryosection obtained from rat liver rapidly clamp-frozen *in situ* with frozen Freon 22. Nuclei (N) and numerous mitochondria (arrows) are indicated, as well as stacks of rough ER (arrowheads). The boxed region is enlarged (inset) to show the stacks of rough ER; some regions show periodic bumps (small arrows) along the membrane which are thought to be ribosomes. The EPMA measurements of the Ca content of ER were obtained from such stacked tubules. Red blood cells (R) are also evident in the liver sinusoids.

Methods. Rats were anaesthetized intraperitoneally with Nembutal (50 mg per kg), an incision made in the abdominal wall over the liver and the rat placed on its abdomen in a cloth hammock above an environmental chamber maintained at 37 °C with high humidity. The liver was exposed gently, without touching its surface, through a slit in the cloth hammock and suspended in the atmosphere of the environmental chamber. The freezing clamp with two opposing aluminium cups filled with solidified Freon 22 was removed from the nitrogen and when the surface of the solid Freon had melted, the door of the environmental chamber was opened and the lobe of the liver clamped rapidly. The temperature of the melted Freon surface is at the melting point of Freon 22 and moulds to the irregular tissue surface, promoting a good contact between the coolant and the tissue. The concept of using melting Freon 'popsicles' originated with Morris Karnovsky (unpublished). The liver was sectioned at -130 °C in a cryoultramicrotome, then freeze-dried³².



of the very small errors of the EPMA measurements. The low Ca content of mitochondria and the significantly higher Ca concentration in the ER are shown clearly in the averaged X-ray spectra in Fig. 2. The concentrations of K, Na, Cl, Mg, P and S, measured in the same regions, are given in Table 1. Estimates of the K concentration in fresh liver obtained by other methods are comparable¹⁴.

The mitochondrial calcium concentration was 0.8 ± 0.1 ($\pm$ s.e.m.) mmol per kg mitochondria ($n=97$; 29 cells, four rats:

two fasted, two fed), which is equivalent to ~ 1.1 nmol per mg mitochondrial protein (ratio of protein to dry mass = 70%). The ratio of free matrix Ca^{2+} to total mitochondrial Ca, in mitochondria isolated from liver, has been estimated as 7×10^{-4} (ref. 15). Given this value, we can estimate that (for 64% mitochondrial H_2O) the free Ca^{2+} concentration in the mitochondrial matrix of rat liver is $\sim 0.3 \mu\text{M}$ (95% confidence limit 0.2–0.4 μM), well within the range (0–2 μM) in which mitochondrial dehydrogenases are regulated. If the mitochondrial Ca concentration

Table 1 Subcellular distribution of calcium and other elements in rat liver parenchymal cells (four animals) (mmol per kg dry wt)

	No. of spectra	Na	Mg	P	S	Cl	K	Ca	% Cell volume*	% Total cell Ca
Mitochondria	97	54 ± 3	43 ± 1	360 ± 7	316 ± 4	75 ± 3	314 ± 5	0.8 ± 0.1	20	5
Rough endoplasmic reticulum	24	80 ± 8	76 ± 2	920 ± 21	224 ± 8	94 ± 5	579 ± 17	5.0 ± 0.4	9.4	$14-23^\dagger$
Nuclei	21	77 ± 6	53 ± 2	557 ± 16	245 ± 8	117 ± 6	625 ± 22	0.8 ± 0.4	6	1
Other regions‡	29	75 ± 6	53 ± 2	653 ± 20	241 ± 9	103 ± 5	465 ± 15	4.0 ± 0.4		
Total cell	20	53 ± 4	46 ± 2	564 ± 12	230 ± 9	106 ± 4	479 ± 20	3.4 ± 0.4		

The instrumentation and methods used for EPMA, including calibration, have been published elsewhere³¹⁻³⁴. To obtain the precision required for these measurements, individual spectra were collected for 400–600 s, until the measurement error of a single spectrum was 1.1 mmol Ca per kg dry wt. Spectra were collected from mitochondria with 75–100-nm diameter probes; from ER with 100-nm or 1,000-nm diameter probes over stacks of ER tubules that included the intervening cytoplasm and, therefore, give an underestimate of the Ca concentration in the ER; from nuclei using probes of diameter 3–10 μm and for total cell measurements, using defocused probes covering whole cell profiles. Values are the mean $\pm$ s.e.m.

* From refs 23, 24.

† The lower value is based on the Ca concentration ([Ca]) of the stacks of rough ER, the higher value on the [Ca] measured with 10–20-nm diameter probes over the ER lumen (see text).

‡ Regions in the cell exclusive of nuclei, mitochondria and stacks of rough ER, which, based on known liver structure, would include the smooth ER tubules that were not imaged in the cryosections.

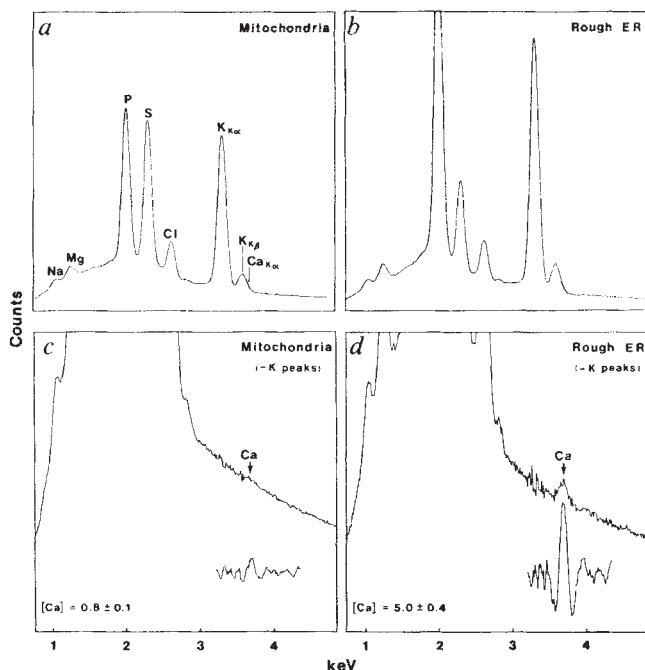


Fig. 2 Averaged X-ray spectra of mitochondria (*a, c*) and rough ER (*b, d*). The Cu background was subtracted from all spectra by the computer. Peaks representing other elements are indicated. In *c* and *d* ($8\times$ higher gain), the potassium K_{α} and K_{β} peaks have also been removed to allow a comparison of the relative sizes of the Ca peaks of mitochondria and ER. The insets show the Ca peaks in the filtered spectra.

were much higher (that is, a total mitochondrial Ca content significantly greater than 2–3 mmol per kg dry wt), the Ca^{2+} regulatory sites on the enzymes would be saturated with Ca^{2+} . Conversely, the mitochondrial Ca content measured by us is insufficient for significant activation of the mitochondrial efflux pathway (apparent $K_m = 9.7 \mu\text{M}$ in isolated liver mitochondria¹⁵), as would be required for effective mitochondrial regulation of cytoplasmic Ca^{2+} (ref. 5). The reported Ca concentrations of isolated liver mitochondria cover a wide range, depending on the isolation conditions (for review, see ref. 5), with recent values between ~ 2 and 17 nmol per mg protein^{10,12,16}. The lowest values have been found in mitochondria isolated in Ca-free media^{10,16}, but we note that in smooth muscle⁹, incubation in Ca-free solutions reduces the mitochondrial Ca content, measured *in situ*, from 1.6 to 0.1 mmol per kg dry weight.

Our findings also have some implications for the reported release of mitochondrial Ca by agents such as phenylephrine, adrenaline or vasopressin¹⁷. The Ca content of mitochondria isolated from phenylephrine-treated, isolated hepatocytes in the latter study was higher (5.0 nmol per mg protein) than we found in the untreated animals *in situ*. Therefore, phenylephrine and similar agents, instead of having an *in vivo* effect on mitochondrial Ca, may merely reduce the translocation of Ca into mitochondria during isolation¹⁰, possibly through the depletion of other sources of intracellular Ca, such as the endoplasmic reticulum.

The concentration of Ca in regions of the endoplasmic reticulum was 5.0 ± 0.4 mmol per kg dry wt; this is very much higher than the concentration in mitochondria or in nuclei (Table 1) but may still be an underestimate, as the electron probe diameters were larger than the diameters of the ER tubules (Fig. 1) in the cryosections. The close-packing of the stacks of tubules of rough ER (Figs 1, 3) over which our measurements were made, suggests that the Ca concentration may have been underestimated by a factor of 2, and that the true Ca content of the rough ER is perhaps 8–10 mmol per kg dry weight. This value is still much higher than the Ca content (~ 2 nmol per mg protein) of microsomes isolated from liver¹¹, but is comparable to the steady-state Ca content of 10 nmol Ca per mg protein

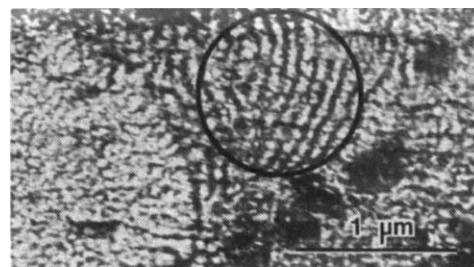


Fig. 3 High-magnification view of a portion of a parenchymal liver cell in an ultrathin cryosection, showing a stacked array of rough endoplasmic reticulum. The encircled region indicates the area that was irradiated with the electron beam during electron probe microanalysis, and which contained the following concentrations ($\pm$ s.d.) of calcium, magnesium and phosphorus: 4.2 ± 0.5 , 87.0 ± 1.5 and 995 ± 4.6 mmol per kg dry wt, respectively.

in microsomes equilibrated with 70 nM free Ca^{2+} (ref. 18).

The high phosphorus (920 ± 21 mmol per kg dry wt) and magnesium (76.1 ± 1.9 mmol per kg dry wt) concentrations in the stacks of rough ER reflect the presence of membrane phospholipids and ribosomes. When probes from a field emission gun were focused (8–20 nm diameter) on ribosomes attached to the stacked ER, the P and Mg contents measured (respectively, $1,129 \pm 77$ and 89.9 ± 3.5 mmol per kg dry wt, $n = 4$) were even higher, confirming that ribosomes^{19,20} make a large contribution to the high P and Mg concentrations in the stacked rough ER. The Ca concentration in the ribosomes was 0.1 ± 0.6 mmol per kg dry wt, and in the lumen of ER tubules (measured with similar small (8–20-nm) probes) it was 8.2 ± 1.9 mmol per kg dry wt ($n = 4$), consistent with the estimate based on measurements made with defocused probes over stacks of rough ER.

The 'cytoplasmic' regions (labelled 'other' in Table 1) that excluded visible organelles (mitochondria, nuclei, stacks of rough ER) had a relatively high Ca content but, based on known liver morphology, they probably also included tubules of smooth and/or rough ER not imaged in unstained cryosections. Therefore, some of the Ca measured in these cytoplasmic regions may be present in the lumen of non-visualized ER, as well as bound to cytoplasmic and membranous proteins such as calmodulin, calelectrin, etc.^{21,22}. The Ca content of nuclei was 0.8 ± 0.4 mmol Ca per mg protein ($n = 21$), providing an upper limit for the Ca that could be bound to small, diffusible cytoplasmic proteins capable of passing through nuclear pores. We were unable to determine the Ca content of individual tubules of smooth ER that were not visualized in the unstained cryosections. EPMA measures total (free and bound) Ca, and our measurements are not directly comparable with values of free and exchangeable Ca measured using other methods.

We determined the subcellular distribution of total liver cell Ca by combining our results with stereological measurements^{23,24} of the fractional volume occupied by various organelles (Table 1); the Ca content of the mitochondria (20% of the cell volume) and of the nucleus (6% of the cell volume) accounts for only $\sim 5\%$ and 1%, respectively, of the 3.4 mmol per kg dry wt total cell Ca. The Ca content of whole fresh rat liver, including extracellular Ca, is reported to be 4.1 mmol per kg dry wt⁵. The minimal fraction of cell Ca in the rough ER, based on a 9.4% volume and on the Ca measurements obtained with defocused probes over the stacks of rough ER, is 14%; this value increases to 23% if the Ca content of the lumen of the rough ER is included. Considering the total cell volume of the ER (15% = 9.4% rough ER and 5.9% smooth ER) and assuming that the Ca concentration in the smooth ER is the same as that in the rough ER, the fraction of the total cell Ca present in the ER could be 23–27%.

Thus, our results support the view^{1,6,7,26} that mitochondrial enzymes may be regulated by matrix free Ca^{2+} , and extend it to a metabolically active, intact, blood-perfused organ. The mitochondrial Ca content is far below the concentration⁵ at

which the mitochondrial Ca efflux pathway is active and mitochondria could effectively regulate cytoplasmic free Ca^{2+} . The high Ca content of the ER is consistent with the view that this is the source of Ca released by inositol trisphosphate²⁷⁻³⁰ and other agents. Our results also show that it is possible to freeze some cells in a near physiological condition *in vivo*, and make direct measurements with EPMA of the composition of organelles at high spatial resolution and sensitivity^{31,32}.

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1. Somlyo, A. P. *Nature* **309**, 516-517 (1984).
2. Martonosi, A. N. in *Muscle and Nonmuscle Motility* Vol. 1 (ed. Stracher, A.) 233 (Academic, New York, 1983).
3. Carafoli, E. & Crompton, M. *Ann. N.Y. Acad. Sci.* **307**, 269-284 (1978).
4. Scarpa, A. in *Membrane Transport in Biology II* (eds Giebisch, G., Tosteson, D. C. & Ussing, H. H.) 263-355 (Springer, New York, 1979).
5. Hansford, R. G. *Rev. Physiol. biochem. Physiol.* (in the press).
6. Denton, R. M. & McCormack, J. G. *FEBS Lett.* **119**, 1-8 (1980).
7. Carafoli, E. in *Exercise Bioenergetics and Gas Exchange* (eds Cerretelli, P. & Whipp, B. J.) 3-12 (North-Holland, Amsterdam, 1980).
8. Somlyo, A. P. & Walz, B. J. *Physiol. Lond.* **358**, 183-195 (1985).

9. Bond, M., Shuman, H., Somlyo, A. V. & Somlyo, A. P. *J. Physiol., Lond.* **357**, 185-201 (1984).
10. Reinhart, P. H., van de Pol, E., Taylor, W. M. & Bygrave, F. L. *Biochem. J.* **218**, 415-420 (1984).
11. Becker, G. L., Fiskum, G. & Lehninger, A. L. *J. biol. Chem.* **255**, 9009-9012 (1980).
12. Joseph, S. K., Coll, K. E., Cooper, R. H., Marks, J. S. & Williamson, J. R. *J. biol. Chem.* **258**, 731-741 (1983).
13. Bookchin, R. M. & Lew, V. L. *Nature* **284**, 561-563 (1980).
14. Howard, R. B., Lee, J. C. & Pesch, L. A. *J. Cell Biol.* **57**, 642-658 (1973).
15. Coll, K. E., Joseph, S. K., Corkey, B. E. & Williamson, J. R. *J. biol. Chem.* **257**, 8696-8704 (1982).
16. Blackmore, P. F., Dehaye, J.-P. & Exton, J. H. *J. biol. Chem.* **254**, 6945-6950 (1979).
17. Babcock, D. F., Chen, J.-L. J., Yip, B. P. & Lardy, H. A. *J. biol. Chem.* **254**, 2117-2120 (1979).
18. Dawson, A. P. *Biochem. J.* **206**, 73-79 (1982).
19. Hamilton, M. G. & Peterman, M. L. *J. biol. Chem.* **234**, 1441-1446 (1959).
20. Siekevitz, P. & Palade, G. E. *J. biophys. biochem. Cytol.* **7**, 631-644 (1960).
21. Sudhof, T. C. *Biochem. biophys. Res. Commun.* **123**, 100-107 (1984).
22. Waisman, D. W., Smallwood, J., Lafreniere, D. & Rasmussen, H. *Biochem. biophys. Res. Commun.* **119**, 440-446 (1984).
23. Weibel, E. R., Staubli, W., Guagi, R. & Hess, F. A. *J. Cell Biol.* **42**, 68-91 (1969).
24. Loud, A. V. *J. Cell Biol.* **37**, 27-46 (1968).
25. van Rossum, G. D. V. *J. gen. Physiol.* **55**, 18-31 (1970).
26. Hansford, R. G. & Castro, F. J. *Bioenerg. Biomembranes* **14**, 361-376 (1982).
27. Berridge, M. J. *Biochem. J.* **212**, 849-858 (1983).
28. Burgess, G. M. *et al. Nature* **309**, 63-66 (1984).
29. Dawson, S. P. & Irvine, R. F. *Biochem. biophys. Res. Commun.* **120**, 858-864 (1984).
30. Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. *J. biol. Chem.* **259**, 3077-3081 (1984).
31. Hall, T. A. & Gupta, B. L. Q. *Rev. Biophys.* **16**, 279-330 (1983).
32. Somlyo, A. V., Gonzalez-Serratos, H., Shuman, H., McClellan, G. & Somlyo, A. P. *J. Cell Biol.* **90**, 577-594 (1981).
33. Shuman, H., Somlyo, A. V. & Somlyo, A. P. *Ultramicroscopy* **1**, 317-339 (1976).
34. Kitazawa, T., Shuman, H. & Somlyo, A. P. *Ultramicroscopy* **11**, 251-262 (1983).

Identification of the human analogue of a regulator that induces differentiation in murine leukaemic cells

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We have recently purified murine granulocyte colony-stimulating factor (G-CSF)¹, a regulatory glycoprotein which stimulates granulocyte colony formation from committed murine precursor cells in semi-solid agar cultures². G-CSF is one of a family of colony-stimulating factors that regulate the growth and differentiation of granulocytes and macrophages³⁻⁵. While the other murine CSFs (granulocyte-macrophage (GM)-CSF, macrophage (M)-CSF and multi-CSF) show little or no differentiation-inducing activity on murine myelomonocytic leukaemia cell lines, G-CSF (or MGI-2⁶) is able to induce the production of terminally differentiated cells from WEHI-3B and other myeloid leukaemia cell lines⁶⁻⁸. More importantly, G-CSF-containing materials suppress the self-renewal of myeloid leukaemia stem cells *in vitro*⁸ and the leukaemogenicity of treated myeloid leukaemic cells *in vivo*⁹⁻¹³. It is important to identify the human analogue of murine G-CSF so that its effectiveness on human myeloid leukaemia cells can be assessed. Here we show that an analogue of G-CSF does exist among the CSFs produced by human cells and that the murine and human molecules show almost complete biological and receptor-binding cross-reactivities to normal and leukaemic murine or human cells. The human G-CSF analogue is identified as a species of CSF that we have previously described as CSF- β (ref. 14).

Two CSFs in human placental conditioned medium that are active on human granulocyte and macrophage progenitor cells have been separated on the basis of differences in hydrophobicity¹⁴; the earlier-eluting species (CSF- α) stimulated the formation of neutrophilic granulocyte, macrophage and eosinophilic granulocyte colonies with colony formation peaking by day 14 of culture, while the later-eluting species (CSF- β) stimulated almost exclusively the formation of neutrophilic granulocyte colonies peaking by day 5-7 of culture. We have confirmed the existence of these two species of human CSF by using medium conditioned by the human bladder cell line 5637 (Fig. 1), as have others using medium conditioned by the GCT cell line¹⁵. After fractionation on phenyl-Sepharose (Fig. 1), CSF- β fractions which were active on human bone marrow cells also

stimulated granulocyte colony formation by murine marrow cells and induced differentiation in murine myelomonocytic leukaemia WEHI-3B D⁺ cells, whereas CSF- α fractions had no such effects. The low frequency, small size and maturity of the murine granulocytic colonies stimulated by CSF- β were typical of colonies stimulated by G-CSF².

In agar cultures of human bone marrow cells, pure G-CSF stimulated granulocytic colony and cluster formation comparable in number, size and rate of development to those stimulated by CSF- β . To minimize possible indirect effects of CSF-producing cells in unfractionated human marrow cultures, we compared the activities of G-CSF and CSF- β in cultures of human marrow cell fractions highly enriched for promyelocytes and myelocytes by fluorescence-activated cell sorting (FACS)¹⁶. The dose-response curves for proliferation in cultures of these cells stimulated by partially purified CSF- α , CSF- β and pure G-CSF were almost identical in shape and, in particular, CSF- β and G-CSF stimulated the development of equal numbers of clones (Fig. 2a). CSF- β and G-CSF also showed similar dose-response curves for inducing differentiation in WEHI-3B D⁺ colonies, while CSF- α was inactive in this assay (Fig. 2b). Interestingly, the dose-response curves for inducing differentiation of WEHI-3B D⁺ colonies were shifted eightfold to the left for both CSF- β and G-CSF compared with the dose-response curves for human progenitor cells (note the different dilutions used in Fig. 2a, b).

¹²⁵I-labelled G-CSF demonstrates specific, saturable binding to WEHI-3B D⁺ leukaemic cells and normal murine bone marrow cells¹⁷. ¹²⁵I-G-CSF also bound specifically to normal human bone marrow and blood cells as well as a variety of fresh human myeloid leukaemic cells (Table 1). The binding of ¹²⁵I-G-CSF to human leukaemias paralleled its specificity of action on normal target cells. Cells from all acute myeloblastic leukaemias, chronic myeloid leukaemias, acute promyelocytic leukaemias and pre-leukaemias showed higher levels of binding than those observed for lymphocytic leukaemia cells. Acute myelomonocytic leukaemias showed relatively little binding of ¹²⁵I-G-CSF compared with the granulocytic leukaemias; this granulocytic specificity of the binding exhibited by ¹²⁵I-G-CSF was confirmed by autoradiography of human bone marrow cells (Fig. 3) which showed that cells of the granulocytic cell lineages were specifically labelled (promyelocytes more intensely labelled than polymorphs) while lymphoid and erythroid cells were not. For comparison, Fig. 3 shows specific labelling of acute promyelocytic leukaemic blast cells.

The binding of ¹²⁵I-G-CSF to WEHI-3B D⁺ cells is inhibited in a dose-dependent manner by unlabelled G-CSF but not by

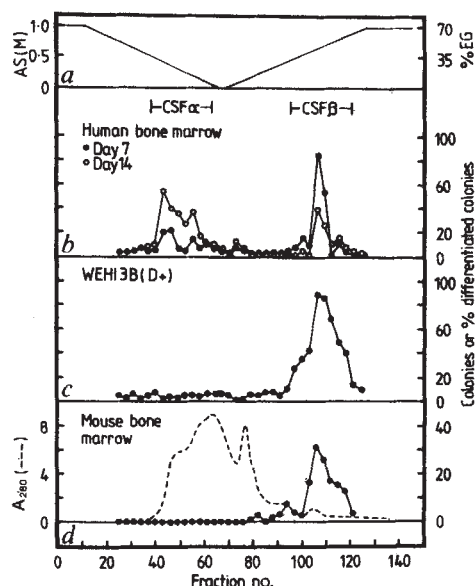


Fig. 1 Separation of human bladder cell line (5637) conditioned medium on phenyl-Sepharose CL-4B. The bladder cell line^{21,22} was incubated at 1×10^6 cells ml^{-1} in DMEM containing 5% FCS at 37 °C in 10% CO_2 in air for 7 days. The conditioned medium was centrifuged at 10,000g for 30 min, made 1 M in ammonium sulphate and applied to a column of phenyl-Sepharose CL-4B (2.6 $\times$ 40 cm) equilibrated in 1 M ammonium sulphate/0.1 M sodium phosphate buffer pH 6.0 containing Tween 20 (0.02%) and sodium azide (0.02%) (start buffer). The column was eluted with a 200-ml linear gradient from start buffer to distilled water, followed by a 200-ml linear gradient from distilled water to 70% ethylene glycol (a) at a flow rate of 20 ml h^{-1} , and 3.5-ml fractions were collected. The fractions were exchanged into phosphate-buffered (20 mM, pH 7.4) saline (0.15 M) containing Tween 20 (0.02%) by passage through Sephadex G-25M and sterilized by passage through Millipore 0.45- μm filters. Aliquots of each fraction were then assayed for their ability to stimulate colony formation of normal bone marrow cells from human rib (100,000 cells per plate), colonies being scored on days 7 and 14 of culture (b); their ability to induce differentiation in colonies formed by murine myelomonocytic leukaemia cells (WEHI-3B D⁺) (300 cells per plate, ~180 colonies) after 7 days of culture (c); and their ability to induce colony formation by normal murine C57BL/6 bone marrow cells (75,000 cells per plate) after 7 days of culture (d). In all cases cultures were prepared in 35-mm Petri dishes containing 0.3% agar, DMEM and 20% FCS and 10% by volume of fraction to be assayed, and were incubated at 37 °C in 10% CO_2 in air in a fully humidified incubator. Colonies comprising >50 cells were scored at $\times 35$ magnification under a dissecting microscope and differentiated WEHI-3B D⁺ colonies were enumerated by their characteristic dispersed morphology. The absorbance of the fractions at 280 nm (dashed line) is also shown in d. AS, ammonium sulphate concentration; % EG, percentage by volume of ethylene glycol in water.

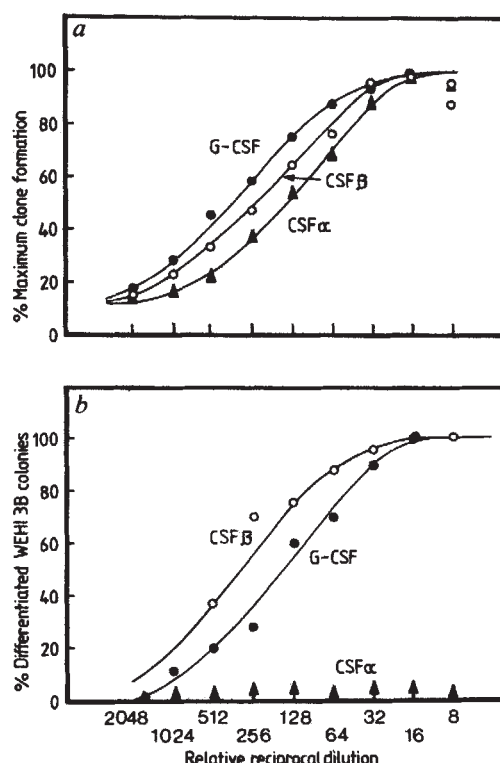


Fig. 2 Ability of human and murine colony-stimulating factors (CSFs) to stimulate clonal proliferation by human cells (a) or to induce differentiation in a murine leukaemic cell line (b). Human CSF- α and CSF- β preparations were from bladder cell line-conditioned medium separated by phenyl-Sepharose CL-4B chromatography (pooled as shown in Fig. 1), and murine G-CSF was pure material from mouse lung-conditioned medium, purified as described elsewhere¹. The human promyelocyte-rich cell fraction (a) was obtained from normal human bone marrow cells after fractionation by FACS using a monoclonal antibody raised against human neutrophils as described elsewhere¹⁶. This fraction contained 35% promyelocytes and myelocytes with no other cells capable of proliferation. Each CSF was titrated in serial twofold dilutions for its ability to stimulate total clone formation (>2 cells) in agar cultures of 2,000 enriched cells as described in Fig. 1 legend, except that clones were scored on day 5 of culture. Maximum clone numbers were 175 for CSF- β or G-CSF and 150 for CSF- α . The distribution of clone sizes was almost identical for CSF- β and G-CSF (2–21 cells) and clones were larger than for CSF- α (2–9 cells). The same pools of CSFs were assayed for their ability to induce a differentiated morphology in murine WEHI-3B D⁺ colonies after 7 days of culture (b). Note that the dilutions of samples in b were eightfold diluted relative to the dilution of samples in a in order to compare more easily the span of the titration curves in the two assay systems.

M-CSF, GM-CSF, multi-CSF or any of several unrelated growth factors¹⁷. Figure 4c shows that human CSF- β inhibited ^{125}I -G-CSF binding to WEHI-3B D⁺ cells to the same extent as unlabelled G-CSF and that the dose-response relationship was indistinguishable from that for G-CSF when the data were standardized to the number of units added, based on the human colony-forming assay of Fig. 2. In contrast, human CSF- α and murine GM-CSF showed no inhibition of binding over the same dose range. Marrow cells of light density from patients with untreated chronic myeloid leukaemia or untreated acute myeloid leukaemia (with >90% leukaemic blast cells) gave binding data that were almost identical to those seen for murine WEHI-3B D⁺ cells (Fig. 4a, b). Moreover, the dose-response relationships for inhibition of ^{125}I -G-CSF binding by unlabelled G-CSF or CSF- β were identical and occurred over the same concentration range as the inhibition of binding to WEHI-3B D⁺ cells while, again, CSF- α and GM-CSF did not inhibit binding. Figure 4d shows that the binding of ^{125}I -G-CSF to BALB/c bone marrow

cells was competed for equally by unlabelled G-CSF and CSF- β and that CSF- α , like GM-CSF, could inhibit only 30% of the specific binding sites; the latter effect appears to result from down-regulation of G-CSF receptors rather than receptor cross-reactivity (N.A.N. and D.M., unpublished).

Saturation binding and Scatchard analyses were performed for the binding of ^{125}I -G-CSF to human acute myeloid leukaemic bone marrow cells (>95% leukaemic blast cells) and murine leukaemic WEHI-3B D⁺ cells (Fig. 5). Because we have noted previously¹⁷ that Scatchard analysis of binding data for WEHI-3B D⁺ cells becomes nonlinear at low levels of bound ^{125}I -G-CSF, the dissociation constants were determined from the limiting slopes of the Scatchard curves; these gave a dissociation constant of 102 pmol for binding to WEHI-3B D⁺ cells and 98 pmol for binding to the acute myeloid leukaemic blast cells. The number of binding sites per cell, estimated from the intercepts on the abscissa, were 300 and 480 for WEHI-3B D⁺ and human acute myeloid leukaemic cells, respectively.

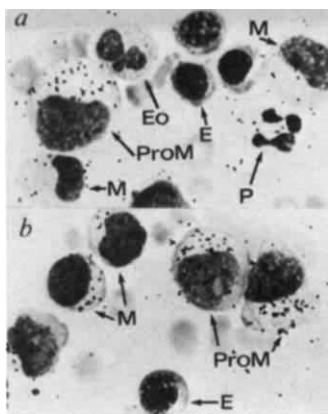


Fig. 3 Autoradiographs of the binding of ^{125}I -G-CSF to: *a*, normal human marrow; and *b*, acute promyelocytic leukaemic marrow. In *a*, note the heavily labelled promyelocyte (ProM) and labelled myelocytes (M) and polymorph (P) but unlabelled eosinophil (Eo) and erythroid cell (E). In *b*, note the labelled promyelocytes and myelocytes but unlabelled erythroid cell. Exposure time 1 month.

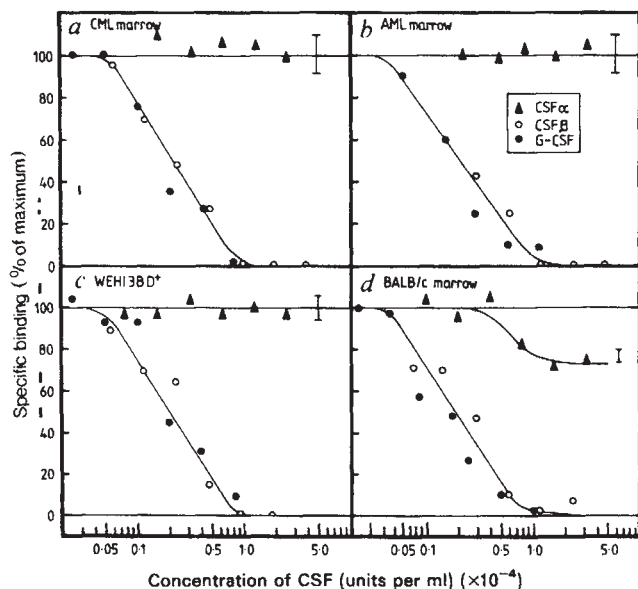


Fig. 4 Ability of human and murine colony-stimulating factors to compete for the binding of murine G-CSF to human and murine cells. Human CSF- α and CSF- β pools and murine G-CSF were obtained as described in Fig. 2 legend and units (U) standardized in the assay on human promyelocyte-enriched cells, as described in Fig. 2 legend (50 U being that amount giving 50% of a maximal clone response). $\circ$, Unlabelled CSF- β ; $\bullet$, unlabelled G-CSF; and $\blacktriangle$, unlabelled CSF- α . Iodinated G-CSF (prepared as described elsewhere¹⁷ (80,000–200,000 c.p.m., specific activity 150,000–300,000 c.p.m. ng^{-1}) and increasing amounts of unlabelled G-CSF, CSF- α or CSF- β as shown were added to 5×10^6 human chronic myeloid leukaemia (CML) bone marrow cells (density $<1.077 \text{ g cm}^{-3}$) (*a*), 5×10^6 human acute myeloid leukaemia (AML) bone marrow cells (density $<1.077 \text{ g cm}^{-3}$) (*b*), 2×10^6 WEHI-3B D⁺ cells (*c*) or 5×10^6 BALB/c murine bone marrow cells (*d*) in DMEM/10% FCS (90 μl final volume) and incubated at 37°C in 10% CO_2 in air for 1 h. Cells were then centrifuged (300g, 5 min) through 200 μl ice-cold FCS, the cell pellet was collected by cutting off the end of the tube, and this was counted in a γ -counter at 76% efficiency. Maximum (100%) specific binding was that in the absence of competitors (mean of duplicate tubes) and 0% specific binding was that in the presence of a 50-fold excess of unlabelled G-CSF (mean of duplicate tubes). The bar on the right-hand side of each panel represents the per cent specific binding seen in the presence of 100,000 U of murine GM-CSF (range for triplicate or more tubes).

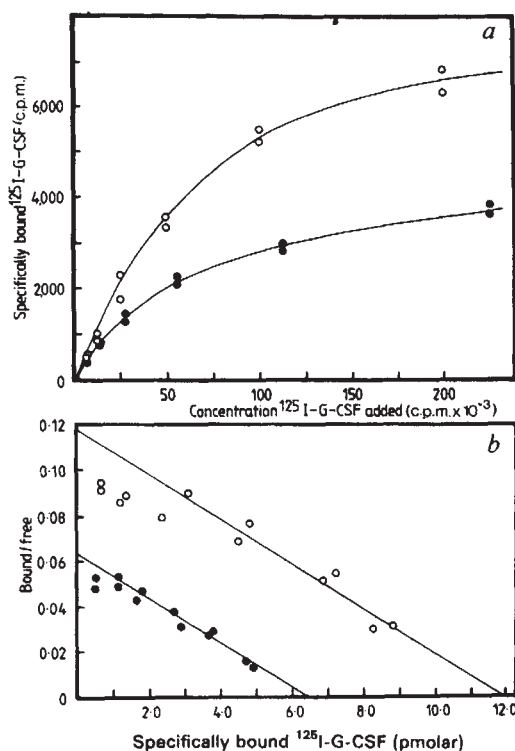


Fig. 5 Saturation analysis of binding of ^{125}I -G-CSF to human AML cells ($\circ$) and murine myelomonocytic leukaemic cell line WEHI-3B D⁺ ($\bullet$). Specific binding was determined as total binding–nonspecific binding; *a* shows the results of duplicate determinations. *b*, The same data converted to Scatchard plots (specific c.p.m. bound/c.p.m. in the supernatants versus the concentration of ^{125}I -G-CSF specifically bound to cells).

Methods. A freshly obtained bone marrow aspirate from a patient with acute myeloid leukaemia classified as M1 was resuspended in DMEM/FCS, layered over Ficoll/Paque (Pharmacia) of density 1.077 g cm^{-3} and centrifuged at 700g for 20 min. The interface cells ($>95\%$ leukaemic blast cells by morphology of stained preparations) were resuspended in DMEM/FCS and passed through a loosely packed, sterile cotton wool plug to remove fat particles. AML cells (2.5×10^6) or WEHI-3B D⁺ cells (2.0×10^6) were incubated with increasing concentrations of ^{125}I -G-CSF as shown, with or without an excess of unlabelled G-CSF (250 ng ml^{-1}) in DMEM/10% FCS (total volume 170 and 160 μl , respectively) at 37°C for 1 h in a fully humidified incubator in 10% CO_2 in air. Tubes were then placed on ice, the cells resuspended and layered over 200 μl cold FCS in 0.5 ml flexible plastic centrifuge tubes and centrifuged at 300g for 5 min. The tubes were then cut with a scalpel blade a few millimetres above the cell pellet and the pellet and supernatants counted separately in a γ -counter. Nonspecific binding (that in the presence of excess unlabelled G-CSF) was linear with respect to the amount of ^{125}I -G-CSF added and was maximally 15% of the specific binding in these experiments.

Most murine haematopoietic growth factors are inactive on human cells but the species cross-reactivity of murine G-CSF and human CSF- β demonstrated here appears to be quite unambiguous. Human CSF- β and murine G-CSF are both hydrophobic molecules of similar size (relative molecular mass 30,000 and 25,000 respectively) and both are able to stimulate granulocyte colony formation by cells from both species with similar dose–response relationships and similar numbers of target cells responsive to either stimulus. That this represents direct cross-reactivity was indicated by the demonstration of specific binding sites for ^{125}I -G-CSF on human myeloid leukaemic cells and the equal abilities of G-CSF and CSF- β to inhibit this binding.

Both molecules also induced differentiation in murine WEHI-3B cells and showed specific binding sites on human myeloid leukaemias. Moreover, CSF- β has been shown to induce differentiation of cells from the human promyelocytic leukaemia cell

Table 1 Specific binding of murine 125 I-G-CSF to human normal and leukaemic cells

Type of patient (F.A.B. classification)	Cell source	No. of patients	Specific binding (% of WEHI-3B D ⁺)	
			Mean ($\pm$ s.d.)	Range
Normal	Bone marrow	5	34 $\pm$ 16	17–55
	Blood buffy coat	4	12 $\pm$ 11	1–27
	Purified blood neutrophils	5	86 $\pm$ 29	45–114
Preleukaemia*	Bone marrow	4	103 $\pm$ 55	62–184
Acute promyelocytic leukaemia (M3) [†]	Bone marrow	2		163, 82
	Blood	1		19
Chronic myeloid leukaemia [‡]	Bone marrow	5	66 $\pm$ 26	27–97
	Blood	3	56 $\pm$ 6	52–63
Acute myeloblastic leukaemia (M1 + M2) [†]	Bone marrow	9	56 $\pm$ 41	18–140
	Blood	9	30 $\pm$ 17	12–56
Acute myelomonocytic leukaemia (M4) [†]	Bone marrow	2		4, 7
	Blood	2		5, 13
Acute lymphoblastic leukaemia [†]	Bone marrow	3	4 $\pm$ 4	1–8
	Blood	1		<1
Chronic lymphocytic leukaemia [§]	Blood	3	4 $\pm$ 6	–1–10

Leukaemic cells of density $<1.077 \text{ g cm}^{-3}$ were prepared by centrifugation over Ficoll/Paque (Pharmacia) at 700g for 20 min. Normal blood cells comprised unfractionated buffy coat cells. Normal granulocytes were purified from blood by density centrifugation in hypertonic metrizamide gradients²⁰ and were 95–100% pure. Cells, usually 5×10^6 per incubation, were incubated in duplicate with 90,000–300,000 c.p.m. of 125 I-G-CSF with or without a 20-fold excess of unlabelled G-CSF in Dulbecco's modified Eagle's medium (DMEM)/ fetal calf serum (FCS) (70–130 μl total volume) at 37 °C for 1 h. Specific binding (total binding – binding in the presence of unlabelled G-CSF) is expressed as a percentage of the specific binding to an equivalent number of WEHI-3B D⁺ cells using the same preparation of 125 I-G-CSF on the same day.

* Including a patient with refractory anaemia with excess blast cells.

[†] Acute leukaemic patients were newly diagnosed or in relapse and their density-fractionated cells contained $>80\%$ leukaemic blasts.

[‡] Cells taken at diagnosis before any treatment.

[§] All showed $>90\%$ leukaemic lymphocytes.

line HL60 (ref. 18). The frequency of binding sites on leukaemic cells was similar to that on the equivalent normal cells and the apparent binding affinity for 125 I-G-CSF appeared to be approximately the same on all the cell types studied. Finally, other studies have shown that G-CSF is able to stimulate various functional activities of post-mitotic neutrophil granulocytes of mouse and man¹⁹.

The strong conservation of G-CSF/CSF- β and their receptors through evolution signals their importance for the maintenance of granulocytic cells in the animal. The conservation of receptors on human granulocytes and myeloid leukaemias indicates that G-CSF and its human analogue, CSF- β , merit further study as regards their possible therapeutic usefulness.

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- Nicola, N. A., Metcalf, D., Matsumoto, M. & Johnson, G. R. *J. biol. Chem.* **258**, 9017–9021 (1983).
- Metcalf, D. & Nicola, N. A. *J. cell. Physiol.* **116**, 198–206 (1983).
- Metcalf, D. *Hemopoietic Colonies* (Springer, Berlin, 1977).
- Nicola, N. A. & Vadas, M. *Immun. Today* **5**, 76–80 (1984).
- Burgess, A. W. & Metcalf, D. *Blood* **56**, 947–958 (1980).
- Lotem, J., Lipton, J. H. & Sachs, L. *Int. J. Cancer* **25**, 763–771 (1980).
- Burgess, A. W. & Metcalf, D. *Int. J. Cancer* **26**, 647–654 (1980).
- Metcalf, D. *Int. J. Cancer* **25**, 225–233 (1980).
- Ichikawa, Y. *J. cell. Physiol.* **76**, 175–184 (1970).
- Fibach, E. & Sachs, L. *J. cell. Physiol.* **86**, 221–230 (1975).
- Honma, J., Kasukabe, T., Okabe, J. & Hozumi, M. *Cancer Res.* **39**, 3167–3171 (1979).
- Metcalf, D. *J. natn. Cancer Inst. Monogr.* **60**, 123–131 (1982).
- Lotem, J. & Sachs, L. *Int. J. Cancer* **28**, 375–386 (1981).
- Nicola, N. A., Metcalf, D., Johnson, G. R. & Burgess, A. W. *Blood* **54**, 614–627 (1979).
- Abboud, C. N., Brennan, J. K., Barlow, G. N. & Lichtman, M. A. *Blood* **58**, 1148–1154 (1981).
- Begley, C. G., Lopez, A. F., Vadas, M. A. & Metcalf, D. *Blood* (in the press).
- Nicola, N. A. & Metcalf, D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3765–3770 (1984).
- Metcalf, D. *Leuk. Res.* **7**, 117–132 (1983).
- Lopez, A. F. *et al. J. Immun.* **131**, 2983–2988 (1983).
- Vadas, M. A., David, J. R., Butterworth, A. E., Pisani, N. T. & Songok, T. A. *J. Immun.* **122**, 1228–1236 (1979).
- Fogh, J. *J. natn. Cancer Inst. Monogr.* **49**, 5–9 (1978).
- Svet-Moldavsky, G. J. *et al. Expl. Hemat.* **8** (Suppl. 7), 76 (1983).

Hybrid antibodies can target sites for attack by T cells

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It would be advantageous in the case of certain diseases to be able to focus a strong T-cell response at a chosen target, for example, in treating cancer or infections that have escaped the normal host response. At present, it seems inconceivable that we could use antigen-specific lines or clones of effector T cells for this purpose because of complications due to the major histocompatibility restriction of T-cell specificity and the problem of rejection of transplanted effector cells. Here we describe a novel technology which combines the power of T lymphocytes in eliminating unwanted cells and causing beneficial inflammatory reactions with the great advantages of monoclonal antibodies (their specificity and availability). We show that heteroconjugates of monoclonal antibodies (referred to hereafter as hybrid antibodies), in which one of the component binding sites is anti-T-cell receptor and the other component binding site is directed against any chosen target antigen, can focus T cells to act at the targeted site. Monoclonal antibodies directed against the T-cell receptor, such as the anti-allotype used here, are mitogenic for resting T cells and can be used to induce effector T cells carrying the T-cell receptor determinant which can then be directed against the target by a hybrid antibody.

Monoclonal antibodies directed against determinants of the T-cell receptor complex of cytotoxic T lymphocytes (CTL) or T-helper cells are able to mimic the effect of antigen in many ways. For example, anti-idiotypes in an appropriate form can

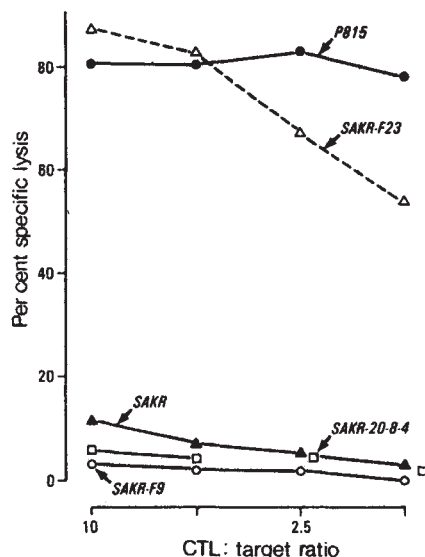


Fig. 1 Anti-receptor allotype monoclonal antibody F23.1 coupled to the target cell surface renders the cell susceptible to lysis by an allotype-positive CTL clone, OE4. F23.1 (IgG2a)⁸, F9 (IgG1) monoclonal antibody specific for an idiotype determinant on CTL clone G4 (ref. 3) and the anti-H-2^b monoclonal antibody 20-8-4 (IgG2a)¹⁵, produced in C3H mice, were purified from ascites fluids on protein A-Sepharose. Antibodies were reacted with the heterobifunctional crosslinker SPDP (Sigma)¹⁶; a threefold molar excess of SPDP was used to introduce two to three groups per 7S antibody. The modified antibody was then reacted with S.AKR lymphoma cells (H-2^k, Thy-1.1) which had been pretreated with 0.5 mM dithiothreitol to introduce free sulphhydryl groups at the cell surface^{17,18}. After washing, 1×10^4 pre-labelled (⁵¹Cr-labelled sodium chromate) target cells were incubated at 37 °C with twofold serial dilutions of the CTL clone OE4 at 37 °C for 4 h (ref. 3). Per cent specific lysis of these target cells and of P815 (DBA/2, H-2^d) targets was calculated as described elsewhere³. The CTL clone OE4 was isolated by limiting dilution from a C57BL/6 (H-2^b) anti-DBA/2 (H-2^d) mixed lymphocyte culture and is maintained by weekly subculture with irradiated DBA/2 spleen cells plus IL-2. OE4 reacts with the anti-allotypic antibody F23.1 and with the anti-H-2^b antibody 20-8-4 but not with the anti-idiotypic F9 antibody. Target cells were: P815 (●), S.AKR (▲), S.AKR coupled to F23.1 (△), S.AKR coupled to F9 (○) and S.AKR coupled to 20-8-4 (□).

stimulate idiotype-positive T-cell clones to release soluble factors such as interleukin-2 (IL-2)^{1,2} or γ -interferon³ (IFN- γ) and to proliferate^{1,2,4}. In addition, recent reports suggest that anti-idiotype on the surface of a cell renders that cell sensitive to recognition and lysis by an idiotype-positive CTL clone: B-cell hybridomas producing anti-idiotype serve as targets for idiotype-positive CTL^{5,6}, and anti-T3 antibodies (T3 is associated with the T-cell receptor) fixed covalently to cells convert them to sensitive CTL targets (H. Spitz, cited in ref. 7).

Recently, we have produced a murine monoclonal antibody specific for an allotypic determinant present on the T-cell receptor of ~25% of peripheral T lymphocytes⁸. The antibody was generated from a C57L/J mouse that had been hyperimmunized with BALB.B peripheral T cells. This monoclonal antibody, F23.1, seems to be very similar in specificity to a rat monoclonal antibody directed against a T-cell receptor isolated from a T hybridoma^{9,10}. To determine whether F23.1, the anti-allotypic monoclonal antibody, could focus CTL onto a target cell, we directly coupled the surface of various cell types to the isolated monoclonal antibody using a heterobifunctional crosslinker. OE4 is a CTL clone of C57BL/6 (H-2^b) origin with lytic specificity for H-2^d-bearing cells. The antigen receptor of clone OE4 bears the allotypic determinant recognized by F23.1. Figure 1 shows that OE4 does not kill cells from the lymphoma S.AKR (derived from AKR/J (H-2^k) mice) in a 4-h cytotoxicity assay, but does efficiently lyse P815 (H-2^d) targets. However, S.AKR

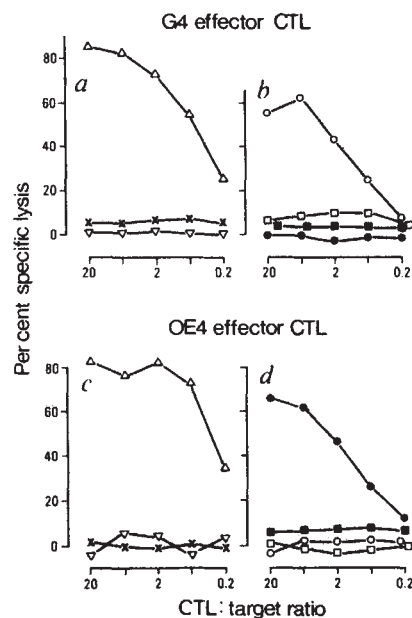


Fig. 2 Heteroconjugates of monoclonal antibodies (hybrid antibodies) can specifically target cells for CTL-mediated lysis. Effector CTL clones were: *a* and *b*, G4 [BALB.B (H-2^b) anti-BALB/c (H-2^d)], which is positive for the idiotype determinant recognized by F9 (ref. 3), and unreactive with F23; *c* and *d*, OE4 [C57BL/6 (H-2^b) anti-DBA/2 (H-2^d)] CTL clone which is F23⁺, F9⁻. Target cells were: P815 (H-2^d, △), S.AKR (H-2^k, ▽), EL4 (H-2^b, ×), S.AKR incubated with 19E12/F23 (●), S.AKR incubated with 19E12/F9 (○), EL4 incubated with 19E12/F23 (■), and EL4 incubated with 19E12/F9 (□).

Methods. The monoclonal alloantibody 19E12 (ref. 11) (IgG2a) specific for the Thy-1.1 alloantigen was purified from ascites fluid on protein A-Sepharose, modified with SPDP¹⁶ and coupled to either F23.1 or F9 monoclonal antibody according to the manufacturer's handbook (Pharmacia). The hybrid antibodies were used to coat ⁵¹Cr-labelled EL4 (H-2^b, Thy-1.2) and S.AKR (H-2^k, Thy-1.1) lymphoma cells by incubation at 37 °C for 20 min followed by washing. These target cells plus uncoated ⁵¹Cr-labelled S.AKR, EL4 and P815 (H-2^d) targets were assayed for lysis by serial threefold dilutions of CTL at 37 °C for 4 h.

cells that had been chemically coupled to F23.1 monoclonal antibody were efficiently lysed by OE4. Target cells coupled to the anti-idiotypic monoclonal antibody F9 (which does not recognize OE4), were insensitive to OE4 effector cells, as were S.AKR cells coupled with an anti-H-2^b monoclonal antibody, 20-8-4, which does react with the H-2^b molecules expressed by OE4. Reciprocal results were obtained with the F9 idiotype-positive, allotype-negative CTL clone G4 (BALB.B (H-2^b) anti-H-2^d). In this case, F9-coupled cells were sensitive targets whereas F23.1- and 20-8-4-coupled cells were not killed (data not shown). Thus, we confirmed that monoclonal antibodies directed against determinants on the T-cell antigen receptor can target cells for attack by CTL when coupled to the surface of the cells. Furthermore, an anti-H-2 monoclonal antibody which reacts with the CTL did not trigger lysis.

We next asked whether heteroconjugates of monoclonal antibodies would be able to specifically target cells for destruction by CTL. In the hybrid antibodies that we constructed, one of the monoclonal antibodies in the hybrid is directed at the T-cell receptor (anti-idiotype or anti-allotype) while the other monoclonal antibody is directed at a surface antigen expressed by the targeted tumour cell but not by the CTL. We chose as the targeting monoclonal antibody 19E12, which is specific for the Thy-1.1 alloantigen¹¹. The CTL clones G4 and OE4 both express Thy-1.2 but not Thy-1.1 and do not react with this monoclonal antibody. The target cell was S.AKR (H-2^k, Thy-1.1). We

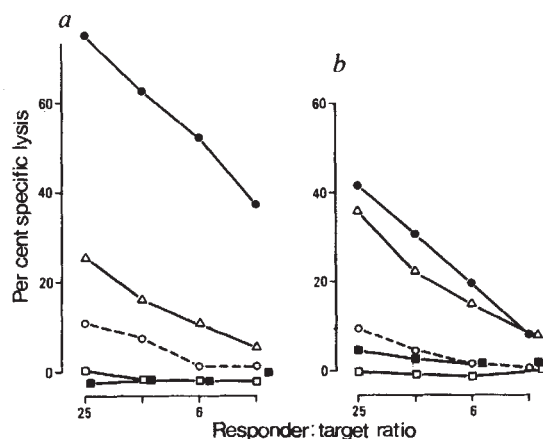


Fig. 3 Cytotoxic effects of Con A-induced (a) and F23.1-induced (b) spleen cells on various targets. Spleen cells from normal BALB.K (H-2^k) mice were cultured for 4 days at 1×10^6 cells ml⁻¹ in RPMI 1640 medium plus 10% fetal calf serum and 10% Con A-induced rat spleen cell supernatant, with either 2.5 μ g ml⁻¹ Con A (a) or F23.1 monoclonal antibody coupled to Sepharose beads (10^3 beads per ml; b). F23.1 cultures also contained 50 mM α -methyl-D-mannoside to block the action of residual Con A present in the rat spleen supernatant. Cells were collected, washed and serial twofold dilutions assayed for lysis of the following ⁵¹Cr-labelled target cells: P815 (○), P815 in the presence of 10 μ g ml⁻¹ PHA (●), AKR.A1 (□), AKR.A1 cells pre-incubated with 19E12 (anti-Thy-1.1)/F23.1 conjugate (Δ), and AKR.A1 cells pre-incubated with the 19E12/F9 conjugate (■). AKR.A1 is a T-cell lymphoma derived from an AKR/J mouse which expresses H-2^k and Thy-1.1.

constructed two hybrid monoclonal antibodies using the cross-linker *N*-succinimidyl-3-(1-pyridyldithio) propionate (SPDP). Both hybrid A (19E12/F9) and hybrid B (19E12/F23) worked efficiently and specifically (see Fig. 2). Hybrid A targeted S.AKR cells for lysis by G4 effector cells (Fig. 2b) while hybrid B targeted S.AKR cells for lysis by OE4 effector CTL (Fig. 2d). Neither hybrid antibody targeted EL4 (H-2^b, Thy-1.2) cells for lysis. Thus, targeting via an antibody combining site works extremely well. Mixtures of unconjugated monoclonal antibodies did not allow target lysis under any circumstances and fractionation of the heteroconjugated monoclonal antibodies on a Sephacryl S-400 column revealed a major peak of specific targeting activity eluting at a relative molecular mass (M_r) of ~300,000, a smaller peak at M_r ~500,000 and no targeting activity at lower or higher M_r ranges (data not shown). Other experiments have shown that EL4 cells, which served as the negative control in Fig. 2, can themselves be targeted for lysis by the syngeneic CTL clone OE4 by first infecting them with vesicular stomatitis virus and using an F23/anti-virus G protein monoclonal antibody heteroconjugate.

With regard to potential therapeutic applications of this hybrid antibody targeting technique, it would be beneficial to be able to generate effector T cells carrying a monoclonal antibody-defined receptor determinant from any individual. The anti-allotypic monoclonal F23.1 reacts with ~25% of peripheral T cells (both CTL and non-cytolytic T cells^{8,12}), is mitogenic for resting, peripheral T cells from mouse strains which are allotype-positive, and induces effector CTL which bear the allotypic determinant (U.D.S. and M.J.B., unpublished). Figure 3 shows the cytotoxic activity of BALB.K (H-2^k) spleen cells that were induced with either the pan-T-cell mitogen concanavalin A (Con A) or with F23.1 monoclonal antibody coupled to Sepharose beads for 4 days in the presence of exogenous IL-2. Con A induces CTL and T-helper cells regardless of their antigen specificity. The CTL activity can be assayed in an antigen-independent fashion by their ability to lyse any target cell coated with an agglutinant such as Con A or phytohaemagglutinin (PHA)¹³. Figure 3a shows that Con A-induced BALB.K cells do not specifically lyse P815 or AKR.A1 (H-2^k, Thy-1.1) targets but do lyse such targets in the presence

Table 1 Monoclonal antibody-induced cytotoxic cells express Thy-1

Target cells	Con A-induced effectors		F23.1-induced effectors	
	C'-treated	Anti-Thy-1+C'	C'-treated	Anti-Thy-1+C'
P815+PHA	76.5	0.4	39.8	-0.6
AKR.A1+19E12/F23	24.2	-0.8	36.7	-0.9

Spleen cells from normal BALB.K mice were cultured for 4 days in the presence of 2.5 μ g ml⁻¹ Con A, or F23.1 monoclonal antibody covalently coupled to Sepharose beads plus 10% supernatant of rat spleen cells which had been cultured with Con A for 24 h as a source of IL-2. Cultures not containing Con A also included 50 mM α -methyl-D-mannoside to block the effect of Con A. Cells were collected and treated with complement (C') alone or with monoclonal anti-Thy-1.2 (ref. 14) plus C', and surviving cells were assayed for 4 h on ⁵¹Cr-labelled target cells. Target cells were P815 in the presence of 10 μ g ml⁻¹ PHA or AKR.A1 cells that had been incubated with a hybrid antibody (19E12/F23) for 20 min and washed. AKR.A1 is a T-cell lymphoma from AKR/J mice which expresses H-2^k and Thy-1.1. Spleen cells cultured without a stimulus had <1% of the cytotoxic activity shown here on either target. Assays were performed at a killer/target cell ratio of 25:1 for the C'-treated cells and for the equivalent number of surviving cells from the anti-Thy-1-treated groups. Values are per cent specific lysis of the target cells³.

of PHA. A fraction of the lytic activity revealed by PHA can also be measured on AKR.A1 cells targeted by the 19E12/F23 hybrid antibody. In the case of F23.1-induced spleen cells (Fig. 3b), PHA plus P815 and the hybrid antibody targeting AKR.A1 cells reveal approximately equal amounts of lytic activity. The levels of lytic activity correlate well with other data showing that the bulk of F23.1-induced T cells express the F23.1 determinant whereas only ~20% of Con A-induced T cells express the F23.1 determinant. As has been shown previously for lectin-induced, lectin-revealed cytotoxicity¹³, all of the monoclonal antibody-induced cytotoxicity is sensitive to treatment with anti-Thy-1 antibody plus complement, that is, is due to T cells (Table 1).

Although we do not know whether target cell destruction in this hybrid antibody-directed system proceeds via the same lytic mechanism as in antigen-specific, CTL-mediated killing, we suspect that the mechanism is very similar for the following reasons: first, the T-cell clones that we used are conventional CTL clones (Thy-1⁺, Lyt-2⁺ cells, whose growth is dependent on a supply of exogenous IL-2 plus periodic antigen stimulation). Second, in the case of Con A-induced effector cells, we have also shown that all of the antibody-directed cytotoxicity is mediated by Lyt-2⁺ T cells. Finally, target cell death in this system has the same kinetics and requirement for cell contact as in antigen-specific CTL-mediated lysis.

The results presented here suggest that it is possible to focus a strong T-cell response to a particular target antigen using hybrid antibodies; this could have clinical significance in the rejection of tumour cells or viral infections. One requirement is an antibody against the chosen target, for example, a tumour-associated or viral antigen; the other requirement is an antibody against the T-cell receptor complex. This technique circumvents the failure of the patients' T cells to respond in an effective way to the antigenic challenge (if any) with a specific, histocompatibility locus antigen (HLA)-restricted response. One can envisage inducing the patient's own T cells *in vitro* with an antibody against a receptor determinant, then transferring them back to the patient in the presence of an anti-target/anti-receptor hybrid antibody. We are presently assessing the *in vivo* applications in a murine lymphoma model system, by growing AKR/J (Thy-1.1) T lymphomas in AKR/Cum (Thy-1.2) hosts and using the hosts' own effector T cells and a 19E12/F23 hybrid antibody.

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- Kappler, J., Kubo, R., Haskins, K., White, J. & Marrack, P. *Cell* **34**, 727-737 (1983).
- Meuer, S. C. *et al. J. exp. Med.* **158**, 988-993 (1983).
- Staerz, U. D., Pasternack, M. S., Klein, J. R., Benedetto, J. D. & Bevan, M. J. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1799-1803 (1984).
- Kaye, J., Porcelli, S., Tite, J., Jones, B. & Janeway, C. A. *J. exp. Med.* **158**, 836-856 (1983).
- Ertl, H. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 7479-7483 (1982).
- Lancki, D. W. & Fitch, F. W. *Fedn. Proc.* **43**, 1659 (1984).
- Martz, E. *Immun. Today* **5**, 254-255 (1984).
- Staerz, U. D., Rammensee, H.-G., Benedetto, J. D. & Bevan, M. J. *J. Immun.* (in the press).
- Haskins, K. *et al. J. exp. Med.* **160**, 452-471 (1984).
- Roehm, N. *et al. Cell* **38**, 577-584 (1984).
- Houston, L. L., Nowinski, R. C. & Bernstein, I. D. *J. Immun.* **125**, 837-843 (1980).
- Staerz, U. D. & Bevan, M. J. *JCSU Short Rep.* **2** (in the press).
- Bevan, M. J. & Cohn, M. *J. Immun.* **114**, 559-565 (1975).
- Marshall-Rothstein, A. *et al. J. Immun.* **122**, 2491-2497 (1979).
- Ozato, K. & Sachs, D. *J. Immun.* **126**, 317-321 (1981).
- Carlsson, J., Drevin, H. & Axen, R. *Biochem. J.* **173**, 723-737 (1978).
- Jou, Y.-H. & Bankert, R. B. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2493-2496 (1981).
- Chen, S.-S. & Katz, D. H. *J. exp. Med.* **157**, 772-788 (1983).

Gene rearrangement in cells with natural killer activity and expression of the β -chain of the T-cell antigen receptor

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The mammalian host defence system can be divided broadly into adaptive and non-adaptive immunity. Adaptive immunity is acquired and is mediated by B and T lymphocytes. Non-adaptive immunity is mediated in part by a small subclass of heterogeneous peripheral blood mononuclear cells. This population, termed null cells, consists of haematopoietic precursors and cells mediating natural killer (NK) activity and antibody-dependent cellular cytotoxicity (ADCC). NK cells are a class of non-adherent, non-phagocytic, rapidly cytotoxic lymphocytes which can efficiently lyse a wide variety of tumour cells¹, virally infected cells² and immature cell types of normal origin³. Despite the broad range of targets, only a limited number of specificities are thought to be involved in target-cell recognition⁴⁻⁶. Morphologically, NK cells are large granular lymphocytes^{7,8}, but they have been shown to exhibit cell-surface markers characteristic of both T cells⁹⁻¹¹ and monocytes¹², raising doubt over their lineage. The recent cloning of the β -chain of the T-cell antigen receptor^{13,14} has now allowed us to investigate whether some NK cells are T-cell-related. We have examined rearrangement and expression of the β -chain of the T-cell receptor in cloned murine NK cell lines and fresh murine NK cell populations, and our results support the hypothesis that a subpopulation of NK cells is related to T cells and provide a basis for examining whether some NK activity is mediated by a small number of T-cell receptors.

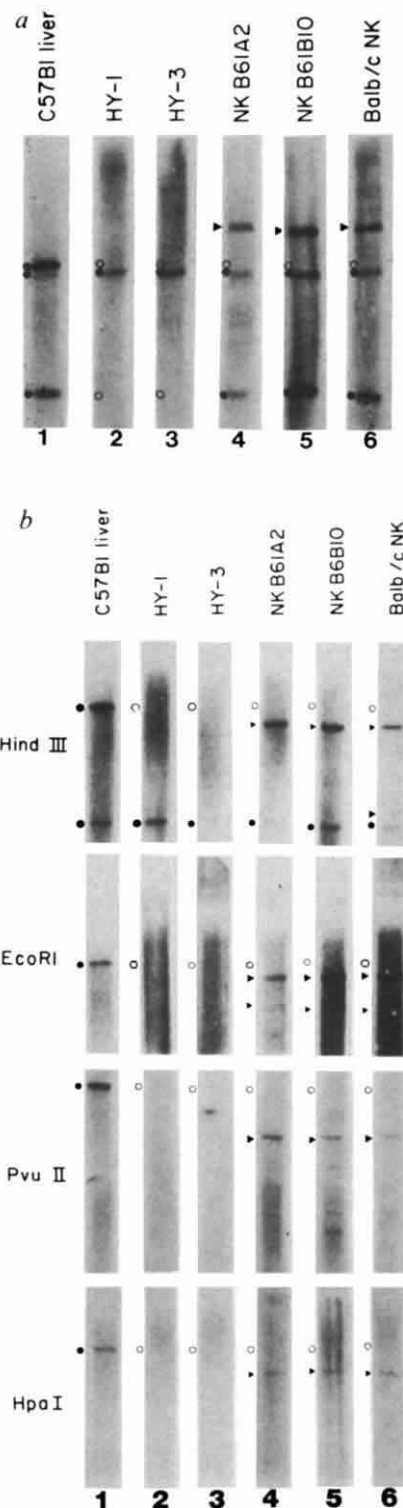
DNA from five independent NK cell lines, HY-1 (ref. 15), HY-3 (ref. 15), NKB61A2 (ref. 16), NKB61B10 (ref. 16) and BALB/c NK (ref. 17), was examined for rearrangement of the genes coding for the β -chain of the T-cell receptor. DNA was digested with six restriction enzymes (*EcoRI*, *PstI*, *PvuII*, *HpaI*, *HindIII* or *XbaI*) and examined by Southern gel analysis using a radioactive probe derived from the joining (J_{β} 1; *EcoRI*, *PvuII*

Fig. 1 *a*, Southern blot analysis of DNA from NK cell lines using restriction enzyme *XbaI*. Lane 1, C57BL/6 liver DNA; lane 2, HY-1 DNA; lane 3, HY-3 DNA; lane 4, NKB61A2 DNA; lane 5, NKB61B10 DNA; lane 6, BALB/c NK DNA. *b*, Southern blot analysis of DNA from NK cell lines using T-cell receptor β -chain constant and joining-region probes. DNA (10 μ g) was digested overnight with a 10-fold excess of *HindIII* (top panel), *EcoRI* (second panel), *PvuII* (third panel) or *HpaI* (bottom panel) (all restriction enzymes from Boehringer Mannheim) and treated as described for *a*, with the exception of the probes. Lane 1, C57BL/6 liver DNA; lane 2, HY-1 DNA; lane 3, HY-3 DNA; lane 4, NKB61A2 DNA; lane 5, NKB61B10 DNA; lane 6, BALB/c NK DNA. Symbols: ●, germline band; ○, bands which have been deleted; ▲, a new, rearranged band.

Methods. *a*, DNA was extracted from the cells using the procedure of Blin and Stafford²⁴. DNA (10 μ g) was digested overnight with a 10-fold excess of *XbaI* (Boehringer Mannheim) and run on a 6-mm 1% agarose gel (Bio-Rad) at 1.0 V cm⁻¹ for 18 h. The DNA was then hybridized with 10⁶ c.p.m. ml⁻¹ (1 μ g of DNA per filter) of nick-translated²⁵ C_{β} 1A probe of the T-cell antigen receptor β -chain genes for 18 h. C_{β} 1A is the 3.5-kb *HindIII*/*SstI* fragment of $C1A$ ¹⁸. This probe contains most of the C_{β} 1 coding domains and some 5'-noncoding sequences. Filters were washed and exposed to Kodak XAR-5 films. *b*, *HindIII*-digested DNA was hybridized to C_{β} 1A probe (described in *a*) and DNA digested with *EcoRI*, *PvuII* and *HpaI* was hybridized to J_{β} 1, the insert from the pUCBJ1 subclone of β -chain genes described by Malissen *et al.*¹⁸.

and *HpaI*) or constant (C_{β} 1; *HindIII*, *HpaI*, *PstI* and *XbaI*) regions of the mouse genomic T-cell receptor β -chain genes¹⁸.

Comparison of the banding pattern of C57BL/6 liver DNA as a control to the NK DNA showed that DNA from all five NK cell lines was rearranged. This result was most evident after digestion by *XbaI* (Fig. 1*a*). There were three germline bands of 5.0, 4.8 and 1.8 kilobases (kb), respectively (Fig. 1*a*, lane 1), and in all cases the 4.8-kb band was retained and the 5.0-kb band deleted. The cell lines HY-1 (Fig. 1*a*, lane 2) and HY-3 (lane 3) had also lost the 1.8-kb band, whereas cell lines



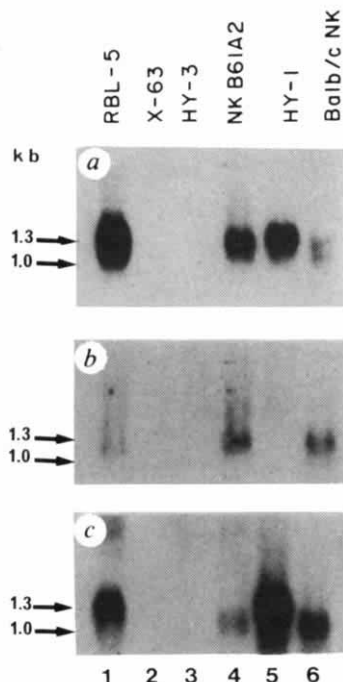


Fig. 2 Northern blot analysis of RNA from NK cell lines using the T-cell receptor β -chain probes. RNA was extracted from cells in the presence of guanidine thiocyanate²⁶ and 10 μ g was treated with glyoxal and electrophoresed through a 1% agarose (Bio-Rad) gel in 10 mM sodium phosphate buffer, pH 7.0. Transfer of the RNA to nitrocellulose filters, hybridization and washing were carried out according to the procedure of Thomas¹⁹. Hybridization was to nick-translated $C_{\beta}1B$ (a), $C_{\beta}1U$ (b) or $C_{\beta}2U$ (c) labelled with ³²P-dCTP according to the procedure of Rigby *et al.*²⁵. $C_{\beta}1B$ is the *EcoRI/EcoRI* insert from the pUC8C1B subclone of the cosmid clone 2.3W7 (ref. 18). $C_{\beta}1U$ is an ~150-base pair *EcoRI/PstI* fragment consisting of the 3'-untranslated region of $C_{\beta}1$, isolated from RBL-5, a murine cDNA clone²⁷. $C_{\beta}2U$ is a 2.5-kb *SstI/SstI* fragment from the cosmid clone 2.3W7¹⁸ which encompasses the 3'-untranslated region of $C_{\beta}2$. Lane 1, RNA from a murine T-cell lymphoma line, RBL-5 (provided by Dr R. Miller); lane 2, RNA from a murine myeloma cell line, X-63 (provided by Dr N. Hozumi); lane 3, HY-3 RNA; lane 4, NKB61A2 RNA; lane 5, HY-1 RNA; lane 6, BALB/c NK RNA.

NKB61A2 (lane 4), NKB61B10 (lane 5) and BALB/c NK (lane 6) had gained a rearranged 6.3-kb band.

We next examined whether either of the two β -chain constant regions was used preferentially in these NK lines. Two probes were used, $C_{\beta}1A$, which hybridizes to both constant regions through the high degree of coding-region homology between them, and $J_{\beta}1$, to confirm that the rearrangement occurs into the first constant region. In the *HindIII* digest hybridized to $C_{\beta}1A$ probe (Fig. 1b, top panel), the 9.8-kb band contained the $C_{\beta}1$ gene whereas the $C_{\beta}2$ gene appeared as a 2.9-kb band (lane 1). In both HY-1 (Fig. 1b, lane 2) and HY-3 (lane 3), the $C_{\beta}1$ band had been deleted and no other band appeared, suggesting that the $C_{\beta}1$ gene has been deleted and that $C_{\beta}2$ is the constant region used. In NKB61A2 (Fig. 1b, lane 4) and NKB61B10 (lane 5), the $C_{\beta}1$ band was replaced by a 7.8-kb band, and in BALB/c NK (lane 6) the $C_{\beta}1$ band was replaced by 7.8- and 3.5-kb bands. *EcoRI*-digested DNA (Fig. 1b, second panel) was hybridized to a $J_{\beta}1$ probe, which does not cross-hybridize to $J_{\beta}2$. The germline band is 9.3 kb (Fig. 1b, lane 1). No hybridization was observed to HY-1 (Fig. 1b, lane 2) or HY-3 (lane 3) DNA, but in NKB61A2 (lane 4), NKB61B10 (lane 5) and BALB/c NK (lane 6), two new bands of 7.3 and 5.0 kb were found in each lane. DNA digested with *PvuII* (Fig. 1b, panel 3) and *HpaI* (Fig. 1b, bottom panel) was hybridized to $J_{\beta}1$. A single germline band of 11.4 kb (*HpaI*) or 5.4 kb (*PvuII*) was replaced by a smaller band of either 9.2 kb (*HpaI*) or 3.3 kb (*PvuII*) in NKB61A2, NKB61B10 and BALB/c cell lines.

Table 1 Phenotypic and functional characteristics of spleen cell subpopulations

Sample	Poly-I:C treatment	Selection treatment	NK lytic units per 10 ⁶ cells	% Lyt-1 ⁺ , 2 ⁺ determined by FACS
A	+	None	10	85
B	—	RFC IgG ⁺	210	2
C	+	RFC IgG ⁺	450	1

Enriched and control populations were tested in a 6-h ⁵¹Cr-release assay against YAC-1.2 to determine lytic unit values (1 lytic unit is the number of effector cells required for 10% lysis). RFC IgG⁺ cells forming rosettes with SRBC coated with anti-SRBC were isolated. FACS activity, fluorescence-activated cell sorting using monoclonal anti-Lyt-1, 2 and fluorescein isothiocyanate coupled with F(ab')₂ goat anti-mouse IgG2b.

Our results indicate that all five NK cell lines have undergone rearrangement of the T-cell receptor β -chain genes and that HY-1 and HY-3 have rearrangements into the $C_{\beta}2$ region, whereas NKB61A2, NKB61B10 and BALB/c NK all rearrange into the $C_{\beta}1$ region, with apparently similar rearrangements. We do not know whether these represent similar rearrangements between variable, diversity (*D*) and *J* regions or only between *D* and *J* regions.

We next examined whether RNA homologous to T-cell receptor β -chain genes is expressed in the NK cell lines. Total RNA was isolated from the five NK cell lines, electrophoresed and hybridized, after blotting, to one of three probes: $C_{\beta}1B$, which contains the coding region of $C_{\beta}1$ and some 5'-noncoding sequences; $C_{\beta}1U$, which is a fragment from RBL5 (ref. 18) containing the 3'-untranslated region of $C_{\beta}1$; or $C_{\beta}2U$, containing the 3'-untranslated region of $C_{\beta}2$.

Figure 2a shows a Northern blot¹⁹ in which $C_{\beta}1B$ was used as a probe; NKB61A2 (lane 4), HY-1 (lane 5), BALB/c NK (lane 6) and NKB61B10 (data not shown) expressed a relatively high level of the β -chain messenger RNA, comparable with that of mouse T-cell lymphoma line RBL-5 (lane 1) used as a control. The B-cell line X-63 (Fig. 2a, lane 2), used as a negative control, expressed no $C_{\beta}1$ -homologous RNA. HY-3 (Fig. 2a, lane 3) expressed a lower level of β -chain mRNA which could be detected only after a longer exposure. Examination of the HY-3 NK cells also revealed a significant decrease in NK killing activity (K.R., unpublished data). Figure 2b, c shows that BALB/c NK and NKB61A2 express a 1.3-kb $C_{\beta}1$ mRNA and a 1.0-kb $C_{\beta}2$ mRNA, whereas HY-1 and HY-3 seem to express only a 1.3-kb $C_{\beta}2$ mRNA. The NKB61B10 line expressed a 1.3-kb mRNA from the $C_{\beta}1$ region (data not shown). In each case, a full-length 1.3-kb mRNA, presumably containing V, (*D*), *J* and *C* sequences²⁰, was detected. Moreover, the constant regions used in these full-length messages agreed with the data on DNA rearrangement. There was no apparent preference for either of the two constant-region genes in these NK cell lines. The lack of correlation between the presence of a 1.0-kb mRNA and the results of the DNA rearrangement analysis is not surprising, as expression of this short mRNA is known to occur only with *D-J* rearrangement^{21,22} or no rearrangement at all²⁰.

To examine whether freshly isolated NK cells also possess these T-cell-like properties, we isolated NK cells from normal mice and from mice stimulated with polyinosinic: polycytidylic acid (poly-I:C). Spleen cells from CBA/J mice were passed over nylon wool columns and NK cells were isolated by recovering cells that form rosettes with sheep red blood cells (SRBC) pretreated with monoclonal IgG2b anti-SRBC antibody. This treatment resulted in a >10-fold increase in the cytolytic activity (lytic units at 10% lysis) against YAC targets and the cells contained <2% Lyt-1⁺, Lyt-2⁺ cells as judged by fluorescence-activated cell sorter (FACS) analysis (see Table 1).

Figure 3 shows Northern blot analysis of RNA from these cells. RNA from spleen cells passed over the nylon wool column hybridized to the $C_{\beta}1B$ probe for the T-cell receptor (Fig. 3a), as the population consists largely (85%) of T cells. The results indicate that enriched NK cells which have not been stimulated

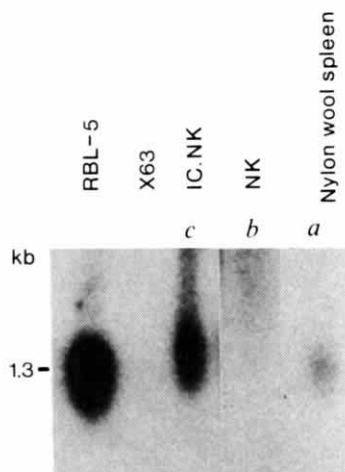


Fig. 3 Northern blot analysis of RNA from NK cells freshly isolated from spleen cells, using T-cell receptor β -chain gene probes. CBA/J mice (4–8 weeks old, $n=100$) were injected intraperitoneally with 100 μ g (0.1 unit) of poly-I:C (Miles Laboratories) per mouse, 24 h before assay (poly-I:C-stimulated mice). A further 100 CBA/J mice were untreated to provide the unstimulated group. Pooled spleen cells were passed over nylon wool columns (a), and enriched for NK cells, by direct rosetting with IgG2b-treated SRBC of unboosted (b) or poly-I:C-boosted cells (c). Nylon-wool-passed cells were rosetted for 1 h at 20 °C with 1% SRBC precoated with 1/50 monoclonal IgG2b anti-SRBC antibody (clone sp2/HL), as described previously²⁸. Rosette-forming cells (RFC) were layered onto Ficoll-Hypaque (1.085 g ml⁻¹) and centrifuged for 20 min at 850g. Lymphocytes were isolated from the pellet by hypotonic shock. RNA was extracted from 3×10^6 cells and Northern blot analysis was carried out as described in Fig. 2 legend.

with poly-I:C contain very little RNA homologous to the T-cell receptor β -chain (after prolonged exposure) (Fig. 3b). However, when poly-I:C-treated spleen cells were enriched and purified for NK cells (Fig. 3c), they were found to contain a mRNA of the same size as that of the positive control samples (RBL-5 RNA and spleen T cells; Fig. 3a). These preliminary results indicate that freshly isolated NK cells, especially poly-I:C-boosted NK cells, may contain mRNA homologous to the β -chain of the T-cell receptor. It is possible that poly-I:C treatment enriched a population of T-cell-like NK cells, but it is unlikely that the presence of the β -chain mRNA can be attributed to contaminating T cells, as they make up less than 2% (one experiment contained no detectable T cells) of the cell population (Table 1).

We demonstrate here that murine NK cells rearrange and express RNA of the T-cell receptor β -chain genes in five murine NK cell lines and in some cells possessing NK activity which were freshly isolated from murine spleens. These results suggest that at least some NK cells are closely associated with the T-cell lineage. Although HY-1 and HY-3 have been identified as cytotoxic T-cell clones, the others were never so identified. The expression of T-cell receptor-homologous mRNA suggests that the protein product of the rearranged genes may be expressed at the NK cell surface. The finding of a recognition structure, NK1a (relative molecular mass 90,000), on a human NK cell line, which seems to be responsible for its cytolytic activity²³, is consistent with these observations. These findings, however, do not suggest that all NK cell activity can be attributed to T-cell-like NK cells or that the T-cell receptor is necessarily responsible for all NK target cell recognition. The data, however, provide a basis for further examination of whether a limited number of functional rearrangements do indeed occur in these NK cells and also if the rearranged T-cell receptor genes code for a receptor that mediates recognition by NK cells.

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- Kiessling, R., Klein, E. & Wigzell, H. *Eur. J. Immun.* **5**, 117–121 (1975).
- Santoli, D., Trinchieri, G. & Leif, F. S. *J. Immun.* **121**, 526–531 (1978).
- Hansson, M., Kiessling, R., Anderson, B., Karre, K. & Roder, J. *Nature* **278**, 174–176 (1979).
- Phillips, W. H., Ortaldo, J. R. & Herberman, R. B. *J. Immun.* **125**, 2322–2327 (1980).
- Allavena, P. & Ortaldo, J. R. *J. Immun.* **132**, 2363–2369 (1984).
- Roder, J. C., Ahlund-Richter, L. & Jondal, M. *J. exp. Med.* **150**, 471–481 (1979).
- Timonen, T., Ortaldo, J. R. & Herberman, R. B. *J. exp. Med.* **153**, 569–581 (1981).
- Timonen, T., Saksela, E., Ranki, A. & Hayry, P. *Cell. Immun.* **48**, 133–148 (1979).
- Perussia, B., Fanning, V. & Trinchieri, G. *J. Immun.* **131**, 223–231 (1983).
- Herberman, R. B., Nunn, M. E. & Holden, H. T. *J. Immun.* **121**, 304–309 (1978).
- West, W. H., Cannon, G. B., Kaye, H. D., Vonnard, G. D. & Herberman, R. B. *J. Immun.* **118**, 355–361 (1977).
- Zarling, J. M. & Kung, P. C. *Nature* **288**, 394–396 (1980).
- Yanagi, Y. *et al. Nature* **308**, 145–149 (1984).
- Hedrick, S. M., Nielson, E. A., Kavalier, J., Cohen, D. I. & Davis, M. M. *Nature* **308**, 153–158 (1984).
- Acha-Orbea, H., Groscurth, P., Lang, R., Stiz, L. & Hengartner, H. *J. Immun.* **130**, 2952–2957 (1983).
- Warner, J. F. & Dennert, G. *Nature* **300**, 31–34 (1982).
- Dennert, G., Yogeewaran, G. & Yamagata, S. *J. exp. Med.* **153**, 545–556 (1981).
- Malissen, M. *et al. Cell* **37**, 1101–1110 (1984).
- Thomas, P. C. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201–5205 (1980).
- Yoshikai, Y. *et al. Nature* **312**, 521–524 (1984).
- Clark, S. *et al. Nature* **311**, 387–389 (1984).
- Siu, G. *et al. Nature* **311**, 344–350 (1984).
- Hercend, T. *et al. J. exp. Med.* **158**, 1547–1560 (1983).
- Blin, N. & Stafford, D. W. *Nucleic Acids Res.* **3**, 2303–2308 (1976).
- Rigby, P., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237–251 (1977).
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294–5299 (1979).
- Caccia, N. *et al. Cell* **37**, 1091–1099 (1984).
- Beaumont, T. J. *et al. Scand. J. Immun.* **16**, 123–133 (1982).

Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus

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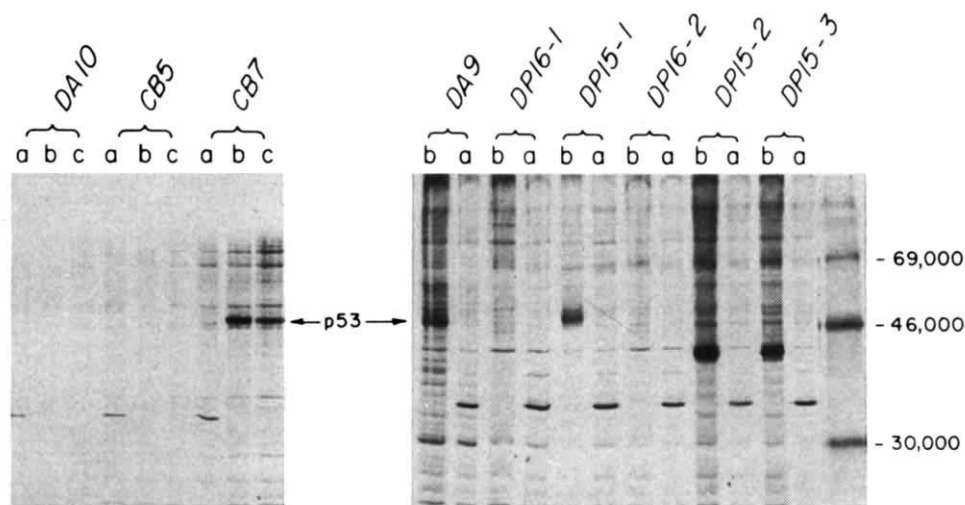
There is now good evidence that the cellular protein, p53, is involved in the transformation process, although its precise role is unknown¹. It was reported recently that expression of the p53 gene can immortalize cells² and that the p53 gene can replace the *myc* oncogene in a *myc-ras* immortalization/transformation assay^{3,4}. We have investigated whether p53 is involved in the progression towards the neoplastic state *in vivo* and report here that erythroleukaemic cell lines transformed by different isolates of Friend leukaemia virus show altered expression of the cellular p53 gene. High levels of p53 protein are found in certain lines, but the protein is undetectable in others. This heterogeneity in p53 gene expression is associated with heterogeneity in tumorigenicity. We demonstrate that genomic rearrangements are responsible for p53 gene inactivation in these cell lines and that they occur *in vivo* during the natural progression of Friend virus-induced erythroleukaemia.

Friend leukaemia virus is a complex of two viruses, a replication-defective spleen focus-forming virus (SFFV) and a replication-competent Friend murine leukaemia virus (F-MuLV)⁵. Clonal cell lines transformed by Friend virus were established from methylcellulose colonies derived from individual spleens of Friend virus-infected mice (see Table 1). To test for the presence of p53 protein in these cell lines, cultures were metabolically labelled with ³⁵S-methionine. p53 was immunoprecipitated from cellular extracts, subjected to electrophoresis in SDS-polyacrylamide gels and visualized following autoradiography (Fig. 1). Most (24/31) of the cell lines expressed p53. Of the remaining seven lines, five did not express detectable p53 protein and two expressed a truncated p53-related protein of relative

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Fig. 1 p53 synthesis in Friend virus-transformed cell lines. Lane a, PAb419 (ref. 6) (control monoclonal antibody); lane b, PAb421 (ref. 6); lane c, RA3-2C2 (ref. 7). Approximately 10^7 cells were labelled for 2 h at 37°C with 0.2 mCi of 35 S-methionine in 0.5 ml α -medium lacking methionine. Cells were then washed with PBS and lysed for 30 min on ice with 0.3 ml lysis buffer (1% Nonidet P-40 (NP40), 150 mM NaCl, 50 mM Tris pH 8). Lysates were cleared by centrifugation for 1 min and by the addition of 0.5 ml of a 10% suspension of formalin-treated *Staphylococcus aureus* Cowan 1 cells (Immunoprecipitin, BRL) for 15 min on ice, followed by centrifugation and retention of the supernatant. Lysate volumes corresponding to equal numbers of trichloroacetic-acid-insoluble counts (10^7 c.p.m.) were immunoprecipitated overnight at 10°C with 40 μ l hybridoma tissue culture supernatant in 400 μ l NET/gel buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.05% NP40, 0.02% sodium azide, 0.25% gelatine). Immune complexes were collected on 50 μ l *S. aureus* cells as above, washed twice in NET/gel buffer and then eluted into 30 μ l sample buffer (2% SDS, 10% glycerol, 0.1% bromophenol blue, 25 mM Tris pH 6.8) by heating at 70°C for 10 min. *S. aureus* cells were removed by centrifugation and samples were loaded onto a 12.5% polyacrylamide gel in the presence of SDS. Electrophoresis was at 35 mA until the blue dye front reached the bottom of the gel. Gels were fixed in 7.5% acetic acid/25% methanol for 30 min before drying and exposure to Kodak XAR-5 film.



molecular mass (M_r) ~44,000. These results were confirmed using other monoclonal antibodies recognizing different epitopes on the p53 molecule. The truncated p53-related peptide was recognized by all the monoclonal antibodies tested (data not shown), PAb421 (ref. 6), RA3-2C2 (refs 7, 8), 200.47 (ref. 9) and PAb122 (ref. 10). Note that the cell lines were derived from the spleens of 10 mice and that some lines may, therefore, be sibling clones. The seven lines showing aberrant expression of p53 were derived from four different mice. Thus, the infected spleens from 4 out of 10 mice gave rise to cell lines displaying aberrant p53 expression.

To estimate the steady-state levels of p53 protein in the various cell lines, p53 was immunoprecipitated from increasing amounts of cell extracts and analysed by the Western blotting technique¹¹. p53 is detected quantitatively by this procedure (Fig. 2A, B). A representative autoradiograph shown in Fig. 2C confirms the

conclusions obtained by metabolic labelling. In cells where p53 protein synthesis was not detected by metabolic labelling, there was at least 100-fold less p53 than in p53-positive Friend cells. p53 was not detected in normal spleen cells (data not shown).

To determine whether the absence of p53 expression, or the synthesis of the antigenically related 44,000- M_r protein, was the result of rearrangement or loss of the cellular p53 gene, genomic DNA from these Friend cell lines was analysed by the Southern blot technique. The probe used, a complementary DNA clone (clone 9 or p27.1a) containing mouse p53-specific sequences^{12,13}, hybridized to 16.0- and 3.3-kilobase (kb) fragments in *Eco*RI-digested normal mouse DNA or DNA from all the Friend lines expressing p53 (Fig. 3C, lanes b, c, f, j). The 3.3-kb DNA fragment has previously been shown to contain a p53 pseudogene¹⁴, whereas the 16.0-kb fragment contains the entire

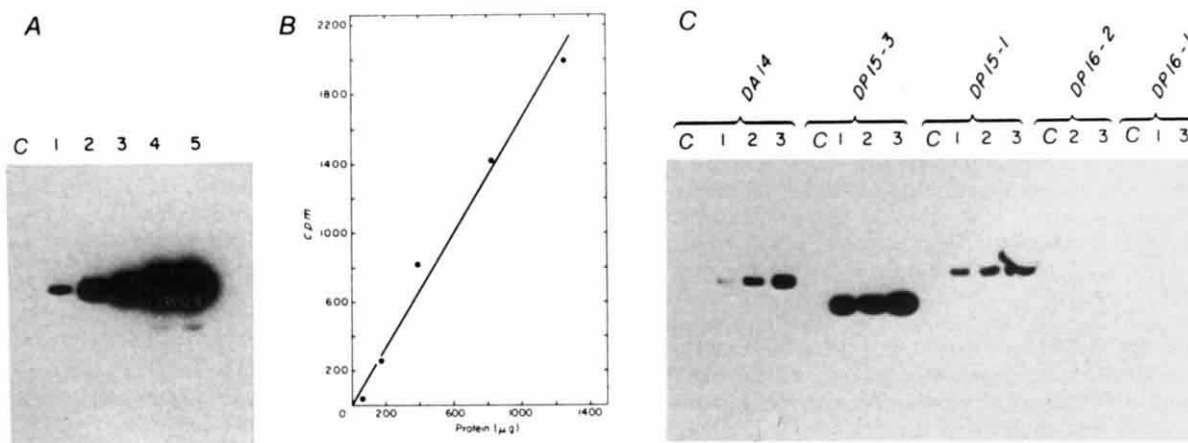


Fig. 2 Quantitative analysis of p53 protein using the Western blotting technique. A, Autoradiograph of the blot. Increasing amounts of cell extract prepared from CB7 cells as in Fig. 1 were immunoprecipitated with an excess of PAb421 monoclonal antibodies against p53⁶. Lanes 1-5 contain 100, 200, 400, 800 and 1,200 μ g of protein, respectively. Lane c contains 400 μ g protein immunoprecipitated with a control monoclonal antibody. The number of 125 I counts bound to the nitrocellulose paper was measured in a γ -counter and used to generate the dose-response curve shown in B. Linear regression analysis was used to fit the best curve to the data. C, Increasing amounts of cell extract prepared from several Friend virus-transformed cells were treated as in A; lanes 1-3 contain 50, 100 and 200 μ g protein, respectively. Lane c contains 100 μ g protein immunoprecipitated with a control monoclonal antibody. Western blot hybridization was performed essentially as described by Burnette¹¹ except that immunoprecipitates, instead of crude extracts, were applied to SDS-polyacrylamide gels and then transferred to nitrocellulose. Electroblothing was carried out at 7-8 V cm^{-1} for 6 h. The nitrocellulose was reacted with anti-p53 antibodies (PAb421) and radioiodinated affinity-purified *S. aureus* protein A (Amersham).

Table 1 Tumorigenicity, spleen colony formation and presence of p53 in various Friend virus-transformed cell lines

Cell lines	Presence of p53 (%)	No. of cells injected	Spleen colonies per 10 ⁵ cells	Tumours/mice injected	Tumour size
DA9	60	10 ⁴	—	6/8	+++
		10 ⁵	26	3/3	+++
		10 ⁶	—	3/3	+++
DA10	<1	10 ⁴	—	8/8	++
		10 ⁵	25	2/3	++
		10 ⁶	—	3/3	++
CB7	100	10 ⁴	—	7/8	+++
		10 ⁵	20	3/3	+++
		10 ⁶	—	3/3	+++
CB5	<1	10 ⁴	—	2/10	++
		10 ⁵	18	—	—
		10 ⁶	—	0/2	—
CB3	<1	10 ⁴	—	1/8	+
		10 ⁵	15	1/3	+
		10 ⁶	—	0/3	—
DP15-1	100	10 ⁴	ND	5/5	+++
DP15-2	<1*	10 ⁴	ND	4/5	+++
DP15-3	<1*	10 ⁴	ND	5/5	+++
DP16-1	<1	10 ⁴	ND	2/5	++
DP16-2	<1	—	ND	ND	—

The cell lines were established from methylcellulose colonies derived from individual spleens of Friend virus-infected mice as described previously¹⁹. Cells were maintained in α -medium supplemented with 10% fetal calf serum. Virus stocks of the polycythaemia- and anaemia-inducing strains of Friend virus complex (FV-P and FV-A, respectively) were obtained as follows. NIH 3T3 fibroblasts non-productively infected with the anaemia-²³ or polycythaemia-²⁴-inducing strains of Friend spleen-focus-forming virus (SFFV_A and SFFV_P) were superinfected with cloned Friend murine leukaemia virus (F-MuLV). F-MuLV was obtained from the tissue culture supernatant of NIH 3T3 cells that had been transfected with F-MuLV DNA clone 57 (ref. 25). Cell lines designated DA and DP were derived from FV-A- or FV-P-infected mice, respectively. The cell lines CB3, CB5 and CB7 were derived from newborn BALB/c mice infected with F-MuLV²⁶. All other cell lines were derived from DBA/2J mice infected as adults with either FV-A or FV-P. Cell lines with the same number, for example, DP15-1, -2, -3, were derived from different methylcellulose colonies from the same mouse spleen. Cell lines CB3 and CB5 were derived from the same mouse. The presence of p53 protein was determined by the Western blotting technique (see Fig. 2 legend). The amounts are relative to cell line CB7. To count spleen colonies, irradiated (850–900 rad) mice were injected with 10⁵ cells in phosphate-buffered saline (PBS). Spleens were removed 9 days later, fixed in Bouin's solution and the colonies counted. The number shown is the average of two spleens. ND, not determined. +++ Refers to large tumours which developed at the site of subcutaneous injection within 2–4 weeks; ++, smaller tumours which developed within 3–6 weeks; +, small tumours which developed 2–3 months after injection.

* These cells contained no detectable p53 protein but did contain high levels of an antigenically related protein of 44,000 *M_r*, that was recognized by monoclonal antibodies to p53 (see Figs 1, 2).

functional p53 gene¹⁵. Similarly, *Bam*HI or *Hind*III digestion of either normal mouse DNA or DNA from p53-producing Friend cell lines resulted in two DNA fragments of 9.0 and 6.0 kb for *Bam*HI and 7.6 and 2.0 kb for *Hind*III which hybridized to the p53 probe (Fig. 3A, lanes b, c, f, k; B, lanes b, d). Thus, at this level of resolution there are no detectable rearrangements of the p53 gene in p53-producing Friend cell lines.

We next examined the seven cell lines that failed to express p53 protein. Digestion of cellular DNA of clone DA10 with the enzymes *Eco*RI, *Bam*HI or *Hind*III revealed no rearrangement of the p53 gene (Fig. 3A–C, lanes a). However, p53 mRNA was not detected by Northern gel analysis¹⁶ (data not shown). Thus, the absence of p53 protein can be explained by a transcriptional block in the expression of the p53 gene. In contrast, the other cell lines contained p53 DNA fragments of different sizes. Cell

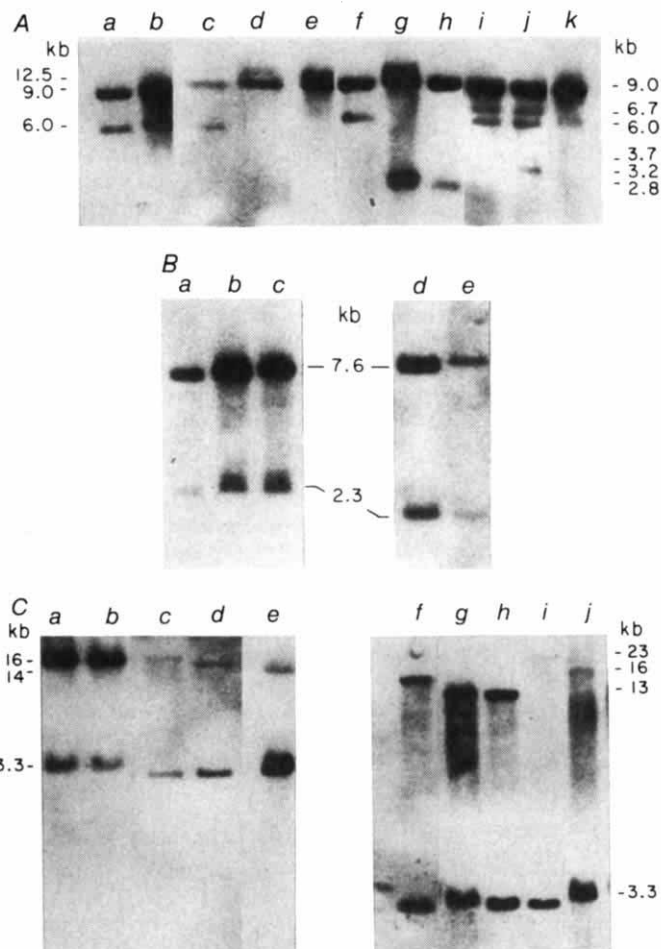


Fig. 3 Genomic DNA blots of Friend virus-transformed cell lines. p53-specific sequences were detected using a p53 cDNA plasmid clone as probe^{12,13}. **A**, Genomic DNA from the following cell lines was digested with *Bam*HI: a, DA10; b, DA9; c, CB7; d, CB5; e, CB3; f, DP15-1; g, DP15-2; h, DP15-3; i, DP16-1; j, DP16-2; k, DP16-3; DP16-3 is a p53-producing cell line. **B**, Genomic DNA from the following cell lines was digested with *Hind*III: a, DA10; b, DA9; c, CB7; d, CB5; e, CB3. **C**, Genomic DNA from the following cell lines was digested with *Eco*RI: a, DA10; b, DA9; c, CB7; d, CB5; e, CB3; f, DP15-1; g, DP15-2; h, DP15-3; i, DP16-2; j, DP16-3.

Methods. DNA was isolated on guanidinium isothiocyanate CsCl gradients²⁷. CsCl (1 g ml⁻¹) was added to the cell lysate in guanidinium isothiocyanate. The lysate was then loaded on a 5.7 M CsCl cushion and spun at 25,000 r.p.m. for 24 h in a SW27 rotor. The viscous DNA above the cushion was dialysed against TE (10 mM Tris pH 8.0, 1 mM EDTA), then extracted with phenol/chloroform. The DNA was precipitated with ethanol, spooled on a glass rod and resuspended in TE and dialysed against TE. DNA was digested and electrophoretically separated in agarose gels as described previously²⁸. The DNA in the gels was acid depurinated by the technique of Wahl *et al.*²⁹, before transfer to zeta probe filters (AMF Corporation) as described by Southern³⁰. The filters were hybridized to 1 × 10⁶ c.p.m. ml⁻¹ of nick-translated³¹ p53 cDNA plasmid^{12,13} in 50% formamide and 10% dextran sulphate for 16 h at 42 °C. The final wash was at 50 °C in 0.1% sodium lauryl sulphate and 0.1 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate).

lines CB5, CB3, DP15-2 and DP15-3 have lost both alleles corresponding to the normal 6.0-kb *Bam*HI fragment (Fig. 3A, lanes d, e, g, h); instead, p53 fragments of ~12.5 kb were observed after *Bam*HI digestion of CB3 or CB5 DNA. Similarly, digestion of DP15-2 and DP15-3 DNA with *Bam*HI produced a p53 DNA fragment of 2.8 kb (Fig. 3A, lanes g, h). Digestion of DNA from these same cell lines with *Eco*RI confirmed both the presence of a rearrangement in the functional p53 gene and the loss of the normal homologous allele (Fig. 3C).

Cell clone DP16-2 also has a rearranged p53 gene, as demonstrated by the loss of the 16-kb *Eco*RI fragment and the appearance of a novel 23-kb DNA fragment (Fig. 3C, lane i). Digestion of DP16-1 and DP16-2 DNA with *Bam*HI resulted in a complex pattern of p53 DNA fragments (Fig. 3A, lanes i, j), suggesting that independent and different genomic rearrangements occurred at each homologous allele to inactivate the p53 gene. Structural alterations in the p53 pseudogene were not observed. We conclude that rearrangement of the p53 gene is involved in gene inactivation and in the production of the truncated 44,000-*M_r* protein.

Because several Friend lines examined here were established from methylcellulose colonies derived from the same mouse, we were able to investigate whether independent *in vitro* colonies were clonally related by examining their unique p53 rearrangements. For example, cell lines DP15-2 and DP15-3 are derived from the same DBA mouse infected with FV-P. These cell lines both express a 44,000-*M_r* protein antigenically related to p53 and have an identical rearrangement of their p53 gene. Similarly, cell lines DP16-1 and DP16-2 share identical rearrangement of their p53 gene (but different from DP15-2 and DP15-3). Cell clones CB3 and CB5 are also derived from the same BALB/c mouse and share identical rearrangement of their p53 gene and common F-MuLV integration sites (data not shown). The presence of unique p53 rearrangements in more than one cell line derived from the same mouse suggests that the genomic p53 rearrangements described here occurred *in vivo* and were not an artefact of cell culture. Thus, we conclude that the genomic rearrangements must have conferred selective advantage on these cells during the natural progression of Friend disease *in vivo*.

It has previously been reported that expression of p53 protein is associated with tumorigenicity in Abelson virus-transformed lymphoid cell lines^{17,18}. The availability of Friend cell lines differing in p53 expression allowed us to investigate whether this heterogeneity was reflected in tumorigenic phenotype. Table 1 compares the ability of the various Friend virus-transformed cell lines to form subcutaneous tumours in syngeneic adult mice. BALB/c mice injected subcutaneously with the p53 producer cell line CB7 had large tumours, whereas no tumours were detected after injection with either of the p53 non-producer cell clones CB3 or CB5. After longer latency periods (1.5–3 months), occasional tumours were observed in these mice. None of the tumours formed by CB3, CB5 or CB7 regressed.

The efficiency of tumour formation by the p53 non-producer cell line DA10 was similar to that observed with the p53 producer cell DA9, although there was a 1–2-week delay in the time when tumours were first detected with DA10. Also, DA10 tumours were much smaller than tumours formed by DA9.

To investigate whether the differences in tumorigenicity between the p53 producer and non-producer Friend cell lines reflected differences in the immunogenicity or *in vivo* growth requirements of these cells, rather than a direct effect of p53 on tumour phenotype, we utilized the fact that Friend virus-transformed cell lines are able to form spleen colonies in irradiated syngeneic hosts^{19,20}. No differences were observed in either the spleen colony-forming efficiency or spleen colony size between p53 producer and p53 non-producer cell lines (Table 1).

To determine whether the tumours that formed from p53 non-producer cell lines were variants or revertants that had now acquired the ability to express p53 protein, we analysed the subcutaneous tumours formed by p53-negative and -positive cell lines by the Western blotting technique. No p53 protein was detected in tumours formed by cell lines DA10, CB3, CB5 and DP16-1, whereas p53 was detected in tumours from cell lines CB7 and DA9 (data not shown). Thus, expression of p53 protein is not essential for tumour formation *in vivo*; rather, the presence

of this protein in Friend virus-transformed cells seems to enhance their tumour phenotype.

Because high levels of p53 protein have been reported previously in four different Friend cell lines²¹, we were surprised to find no p53 expression in cell lines derived from the infected spleens of three mice. The cell lines used in the previous study were all derived after subcutaneous passage of transplantable tumours whereas the Friend virus-transformed cell lines examined in our study were from colonies obtained by plating spleen cells from mice infected with Friend virus in methylcellulose. Thus, unlike the earlier study²¹, there was no direct selection for tumorigenicity placed on the cell lines used here.

The high frequency of p53 gene inactivation and rearrangement observed in Friend erythroleukaemic cells is intriguing and unexpected. Moreover, it suggests that inactivation of the p53 gene is not a random event, but is closely associated with the induction of erythroleukaemia by Friend virus complex or its helper virus. Note, however, that in the p53-producing cell lines examined here, the amount of p53 protein is at least 100-fold higher than in normal spleen cells. Thus, transformation of erythroid cells by Friend virus is accompanied by major changes in the regulation of p53 protein levels—p53 protein levels may rise ~100-fold or expression of the p53 gene may be completely shut-off through gene inactivation. In the latter case, neither cell division nor immortalization functions seem to be affected.

We cannot explain why many tumour cells express high levels of p53 protein whereas some of the Friend cell lines described here, as well as an Abelson virus-transformed cell line described previously²², have genomic rearrangements leading to p53 gene inactivation. Resolution of this paradox may provide some insights into the roles of p53 in normal and malignant cells.

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1. Crawford, L. *Int. Rev. exp. Path.* **25**, 1–50 (1983).
2. Jenkins, J. R., Rudge, K. & Currie, G. A. *Nature* **312**, 651–654 (1984).
3. Parada, L. F., Land, H., Weinberg, R. A., Wolf, D. & Rotter, V. *Nature* **312**, 649–651 (1984).
4. Eliyahu, D., Raz, A., Gruss, P., Givol, D. & Oren, M. *Nature* **312**, 646–649 (1984).
5. Teich, N. *et al.* in *RNA Tumor Viruses* (eds Weiss, R., Teich, N., Varmus, H. & Coffin, J.) 857–880 (Cold Spring Harbor Laboratory, New York, 1982).
6. Harlow, E., Crawford, L. V., Pim, D. C. & Williamson, N. M. *J. Virol.* **39**, 861–869 (1981).
7. Coffman, R. L. & Weissman, I. L. *J. exp. Med.* **153**, 269–279 (1981).
8. Rotter, V., Boss, M. A. & Baltimore, D. *J. Virol.* **38**, 336–346 (1981).
9. Dippold, W. G., Jay, G., Deleo, A. B., Khoury, G. & Old, L. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1695–1699 (1981).
10. Gurney, E. G., Harrison, R. O. & Fenno, J. *J. Virol.* **34**, 752–763 (1980).
11. Burnette, W. N. *Analyt. Biochem.* **112**, 195–203 (1981).
12. Benchimol, S. *et al.* in *Cancer Cells Vol. 2* (eds Levine, A., Topp, W., Vande Woude, G. & Watson, J. D.) 383–391 (Cold Spring Harbor Laboratory, New York, 1984).
13. Jenkins, J. R., Rudge, K., Redmond, S. & Wade-Evans, A. *Nucleic Acids Res.* **12**, 5609–5626 (1984).
14. Zakut-Houri, R. *et al.* *Nature* **306**, 594–597 (1983).
15. Bienz, B., Zakut-Houri, R., Givol, D. & Oren, M. *EMBO J.* **3**, 2179–2183 (1984).
16. Thomas, P. S. *Methods Enzym.* **100**, 255–266 (1983).
17. Rotter, V., Abutbul, H. & Wolf, D. *Int. J. Cancer* **31**, 315–320 (1983).
18. Wolf, D., Harris, N. & Rotter, V. *Cell* **38**, 119–126 (1984).
19. Mager, D. L., Mak, T. W. & Bernstein, A. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1703–1707 (1981).
20. Mager, D. L., Mak, T. W. & Bernstein, A. *Nature* **288**, 592–594 (1980).
21. Ruscetti, S. K. & Scolnick, E. M. *J. Virol.* **46**, 1022–1026 (1983).
22. Wolf, D. & Rotter, V. *Molec. cell. Biol.* **4**, 1402–1410 (1984).
23. MacDonald, M. E. *et al.* *J. exp. Med.* **151**, 1477–1492 (1980).
24. Bernstein, A., Mak, T. W. & Stephenson, J. R. *Cell* **12**, 287–294 (1977).
25. Oliff, A. *et al.* *J. Virol.* **33**, 475–486 (1980).
26. Shibuya, T. & Mak, T. W. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3721–3725 (1983).
27. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294–5299 (1979).
28. Mowat, M. & Bernstein, A. *J. Virol.* **47**, 471–477 (1983).
29. Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683–3687 (1979).
30. Southern, E. J. *molec. Biol.* **98**, 503–517 (1975).
31. Rigby, P. W. J., Diekmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237–251 (1977).

α -Interferon-induced transcription of HLA and metallothionein genes containing homologous upstream sequences

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Complementary DNAs corresponding to the interferon (IFN)-induced messenger RNAs for histocompatibility locus antigens (HLA), metallothionein-II (MT2), 2',5'-oligoadenylate synthetase and about eight other proteins of unknown sequence have been isolated recently¹⁻⁵, and by interferon regulation of transcription has been demonstrated for several of the eight mRNAs^{3,4}, with a significant increase apparent in as little as 5 min³. We now show that IFN- α treatment results in a three- to fivefold increase in the transcription of MT2 and HLA class I genes in human T98G neuroblastoma cells. Furthermore, comparison of regions upstream of the MT2A gene, two HLA genes and one HLA class II gene reveals a homologous sequence of ~30 base pairs (bp) which may be involved in regulating transcription of interferon-induced genes. Transcription of the mRNA for human MT2A is induced by glucocorticoids or metal ions^{6,7} and the regulatory elements have been mapped by promoter-fusion experiments⁸. We now show that the rate of transcription of MT2A is the same on treatment with interferon or dexamethasone, but that the mRNA accumulates much faster with dexamethasone, indicating that post-transcriptional events are important in the latter case.

We determined relative rates of transcription in isolated nuclei by hybridizing ³²P-labelled RNA, synthesized in run-off transcription reactions, to cDNA probes bound to nitrocellulose filters. The assay measures the relative number of transcriptional complexes initiated on genes corresponding to the complementary DNA probes (see ref. 3 for experimental details and discussion of this method). To measure transcription from HLA class I genes, we used plasmid pABC001 (ref. 9), containing HLA-B7 cDNA that hybridizes to HLA-A, HLA-B and HLA-C mRNAs. The 1-8 and MT2 cDNAs used were isolated by Friedman *et al.*³. A full-length human β -actin cDNA¹⁰ was used as a control in all experiments.

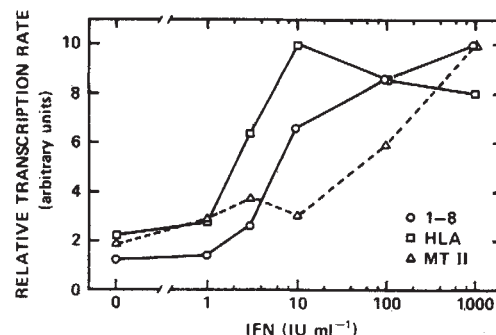


Fig. 1 Dependence of transcription on IFN- α concentration. T98G cells were treated with IFN- α for 2 h and relative transcription rates were measured in the nuclear run-off assay. Duplicate assays agreed to $\pm 20\%$. To facilitate comparisons, the highest point in each assay was assigned a relative rate of 10. T98G human neuroblastoma cells were grown as described previously³. Well-feron, a highly purified mixture of IFN- α , specific activity $1-2 \times 10^8$ IU ml⁻¹ (ref. 23), was used in all experiments. Transcription assays were modified slightly from those used by Friedman *et al.*³ in that hybridizations were carried out in a volume of 300 μ l, containing 1.5×10^7 c.p.m. of ³²P-labelled RNA.

Interferon-induced transcription of 1-8, HLA and MT2 in a dose-dependent manner (Fig. 1), while transcription of the β -actin gene was not affected by any IFN- α concentration tested. Half-maximal induction of MT2 transcription required 30 times more IFN- α than similar induction of HLA genes. We have shown previously that the accumulation of six mRNAs is a function of interferon concentration over a wide range and that the concentration of IFN- α for half-maximal induction is different for different mRNAs. The accumulation of MT2 mRNA requires a higher concentration of IFN- α than any of the others³. We conclude that dose-dependent changes in transcription rate account, at least in part, for the dependence of mRNA accumulation on interferon concentration.

When cells were treated for 13 h with 1,000 IU ml⁻¹ IFN- α , the transcription rates of 1-8, MT2 and HLA genes were about half those observed after 2 h of treatment (Fig. 2a). For the 1-8 gene, the extent of this decrease was less than we reported previously³ at a lower IFN- α concentration (100 IU ml⁻¹), suggesting that the degree of down-regulation of 1-8 transcription may depend on the concentration of IFN- α . We used 1,000 IU ml⁻¹ of IFN- α in the current experiments to obtain maximum induction of MT2.

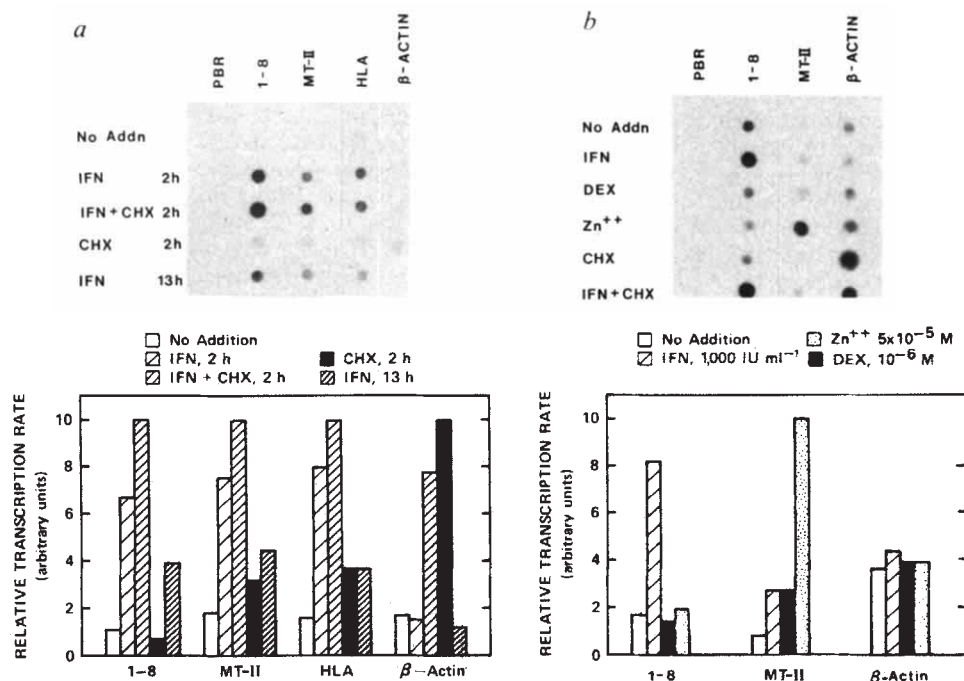


Fig. 2 *a*, Effect of inhibiting protein synthesis or prolonging treatment with IFN- α on interferon-induced transcription. The results of duplicate experiments were averaged. The values agreed to $\pm 15\%$ except for HLA genes in cycloheximide (CHX)-treated cells, where the duplicates agreed to $\pm 50\%$. 1-8 and β -actin served as positive and negative controls, respectively. The concentration of IFN- α was 1,000 IU ml⁻¹. Cycloheximide was used at 32 μ g ml⁻¹, a concentration that inhibits protein synthesis in T98G cells by $>95\%$ ³. *b*, Effects of IFN- α (1,000 IU ml⁻¹), dexamethasone (DEX, 1 μ M) and Zn²⁺ (50 μ M) on transcription. Treatment was for 2 h. The results of duplicate experiments, which agreed to $\pm 15\%$, were averaged. The highest point for β -actin was assigned a relative value of 5.

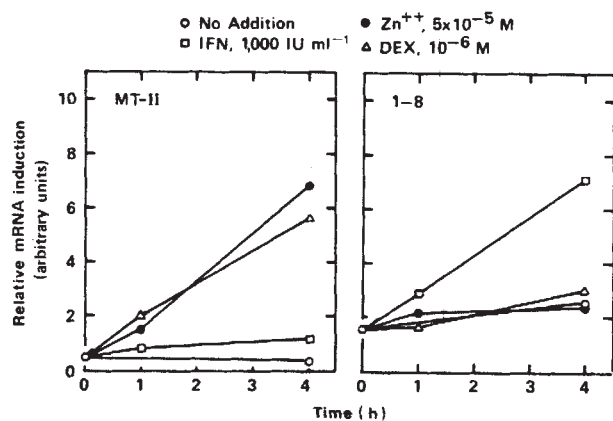


Fig. 3 Effects of IFN- α , dexamethasone and Zn^{2+} on accumulation of *MT2* mRNAs. Levels of mRNA were measured by the slot hybridization technique described previously³. One experiment is shown, but similar results were obtained in two additional independent experiments. The increase in *MT2* mRNA after 4 h was 3–5-fold for IFN- α , consistent with the results reported previously³ and 15–20-fold for Zn^{2+} or dexamethasone. Rabbit β -globin mRNA was added to the lysis buffer to allow correction for differences in RNA recovery to be made. Levels of endogenous β -actin mRNA were also measured. The β -actin and β -globin mRNA levels did not vary significantly with any of the treatments used.

Pretreatment with cycloheximide did not block interferon-induced transcription of the *MT2* or *HLA* genes (Fig. 2a), in agreement with our previous result³ for 1-8. Cycloheximide alone had little effect on the transcription of these genes, but greatly increased transcription of the β -actin gene, as reported previously^{3,11}.

The increased transcription of *MT2* after treatment with IFN- α (Fig. 2a) shows that control of the *MT2* promoter is more complex than believed previously⁸. To define regulation of *MT2* expression more precisely, we measured mRNA levels and transcription rates following exposure of the cells to IFN- α , dexamethasone or Zn^{2+} ; we found that either IFN- α or dexamethasone induced a 3–4-fold increase in the transcription rate of *MT2* (Fig. 2a,b) while treatment with 50 μ M Zn^{2+} produced a 13-fold increase (Fig. 2b).

The rate of *MT2* mRNA accumulation was linear for 4 h after treatment with IFN- α , dexamethasone or Zn^{2+} (Fig. 3). We have shown previously³ that the induction of *MT2* mRNA by interferon is maximal after 12 h. In control experiments, neither dexamethasone nor Zn^{2+} stimulated accumulation of 1-8 mRNA (Fig. 3). Although IFN- α or dexamethasone produced similar increases in *MT2* transcription (Fig. 2b), the accumulation of *MT2* mRNA was much greater with dexamethasone (Fig. 3). Treatment with Zn^{2+} increased gene transcription much more than did dexamethasone treatment (Fig. 2b), but the rates at which the mRNAs accumulated were very similar (Fig. 3). We conclude that post-transcriptional stabilization and increased transcription both contribute significantly to accumulation of *MT2* mRNA in dexamethasone-treated T98G cells. This effect is selective, because treatment with dexamethasone does not lead to an increase in 1-8 or β -actin mRNA.

Yoshie *et al.*¹² transfected Ltk⁻ cells with fragments of an *HLA* gene lacking not only the 5'-flanking sequences but also all of the first and part of the second exon. Nevertheless, RNAs corresponding to the fragments were induced on treatment with 1,000 IU ml⁻¹ of IFN- α . As shown in Figs 1 and 2, IFN- α induces transcription of class I *HLA* genes, so it is possible that signals for interferon-induced transcription are preserved in the gene fragments of Yoshie *et al.*¹². However, we have shown previously that post-transcriptional events can contribute to accumulation of the interferon-induced 1-8 mRNA³. Therefore, it seems more likely that the results of Yoshie *et al.*¹² are explained by interferon-induced post-transcriptional changes and that the transcription rates of the deleted, transfected *HLA* gene fragments do not change when interferon is added.

HLA-DR	GTGTTGAACCTC-AGAGTTTCTCTCT-CAT
HLA-A3	ATTCCCCAAGCTCCGAGTTTCTTTCT-CTC
HLA*	ATTGCCCACCTCCGAGTTTCTCTCTCTCTC
MT-II	TCTCTGGACCT--GCAGTTTCTCTCTCT-CTA
Consensus	TTCTG ^N ACCTCNGCAGTTTCTCTCTCT-CT

Fig. 4 Homologous 5' sequences flanking the *MT2* and *HLA* genes. Bold face letters indicate nucleotides conserved in at least three of the four examples. N, any nucleotide. The sequences are from Malissen *et al.*¹⁶, Karin and Richards²⁴ (with one modification, provided by Dr Michael Karin, personal communication), Das *et al.*¹⁷ and Strachan *et al.*¹⁵. Access to the QUEST, SEQ and IFIND programs was generously provided by IntelliGenetics, a division of IntelliCorp. QUEST was used to retrieve sequences from the NIH and EMBL databases. SEQ was used to find initial intra- and intersequence homologies, for probability calculations and for symmetry searches. The default SEQ parameters were used except for Loopout, which was set to 1. IFIND, based on the algorithm of Wilbur and Lipman²⁵, was used to make the final alignments, to arrive at a consensus sequence, and to search the NIH and EMBL databases for homologies to the consensus sequence.

Our conclusion that post-transcriptional events are significantly involved in the accumulation of *MT2* mRNA after dexamethasone treatment is similar to that of Vannice *et al.*¹³, who showed that dexamethasone produces a marked accumulation of the mRNA for α_1 -acid glycoprotein, but no increase in transcription. We suggest a refinement of the idea of Richards *et al.*¹⁴ that differences in sequences 5' to *MT2* genes account for their differential regulation. Sequences responsible for post-transcriptional regulation are probably also involved.

Because IFN- α regulates transcription of the *MT2* and *HLA* genes, we compared the sequences upstream from *MT2* with sequences upstream from the three *HLA* genes for which such information is available—a class I *HLA-A3* gene¹⁵, an unidentified class I gene¹⁶ and a class II *HLA-DR* gene¹⁷. Class I genes are efficiently induced by both IFN- α and IFN- γ , whereas the *HLA-DR* gene is efficiently induced by IFN- γ and poorly induced by IFN- α . The cap sites for *HLA-A3* and the gene of Malissen *et al.*¹⁶ have not been mapped. By analogy to the *HLA-DR* sequence, we tentatively assign the CAT (CCAAT) and TATA (TCTAAA) boxes to positions 162 and 188 in the *HLA-A3* sequence and to positions 156 and 182 in the sequence of Malissen *et al.*¹⁶.

The two *HLA* class I sequences share considerable homology upstream of their 5' start sites. There is also homology between a region about 760 bp 5' to the start site of the *MT2A* gene and a region about 250 bp 5' to the putative start site in the sequence of Malissen *et al.*¹⁶ (Fig. 4). As calculated with the SEQ program, the probability of finding such a homology by chance is 0.018, taking the base compositions into account. A similar region of homology was also found for *HLA-A3* and *HLA-DR* (Fig. 4). The only significant homology between sequences 5' to the two *HLA* class I genes and the class II *HLA-DR* gene was found in the region also homologous to *MT2*. Alignment reveals that AGTTTCTCTCTC, the core of the consensus sequence, is nearly invariant and that the first seven bases of this core are absolutely invariant. The consensus sequence lies 142 or 147 bp upstream of the TATA box of the class I gene of Malissen *et al.*¹⁶ or the *HLA-A3* gene, respectively. It is 600 or 567 bp upstream of the *MT2* or *HLA-DR* TATA box, respectively, and there is no dyad symmetry near any of the consensus sequences. We also compared the consensus sequence with all human and murine sequences in the databases of the National Institutes of Health (NIH) and the European Molecular Biology Laboratory (EMBL). Only the *HLA* and *MT* sequences used to derive the consensus showed significant homology to it, indicating that the consensus is not merely a sequence repeated frequently in genomic DNA.

The consensus sequence extends over 28 bp and is considerably longer than the sequences proposed to be involved in glucocorticoid-mediated induction of mouse mammary tumour

virus (TGGTACAAAATGTTCT) or *MT2* transcription^{8,14}, or in metal ion induction⁸ of *MT2* ($\text{GTGCGCCCGGC}^{\text{TC}}$). It is also more extensive than the consensus sequence for transcriptional regulation of heat shock genes (GTNGAANNTTCNAG) which has been shown to function in mammalian cells, *Xenopus* oocytes and *Drosophila*¹⁸⁻²⁰. General regulation of yeast amino-acid biosynthesis also depends on a short regulatory sequence ($\text{A}^{\text{A}}\text{GTGACTC}$)^{21,22}. The sequences involved in regulation of transcription by glucocorticoids, metal ions or heat shock are conserved among different species. We have found no homology between the consensus sequence shown in Fig. 4 and sequences 5' of mouse *H-2* genes, but the mouse sequences available in the NIH and EMBL databases do not extend far beyond the 5' start sites of the mRNAs.

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1. Chebath, J., Merlin, G., Metz, R., Benech, P. & Revel, M. *Nucleic Acids Res.* **11**, 1213-1226 (1983).
2. Merlin, G., Chebath, J., Benech, P., Metz, R. & Revel, M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4904-4908 (1983).
3. Friedman, R. L., Manly, S. P., McMahon, M., Kerr, I. M. & Stark, G. R. *Cell* **38**, 745-755 (1984).
4. Larner, A. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 6733-6737 (1984).
5. Rosa, F. *et al. EMBO J.* **2**, 1583-1589 (1983).
6. Karin, M., Andersen, R. D., Slater, E., Smith, K. & Herschman, H. R. *Nature* **286**, 295-297 (1980).
7. Karin, M., Andersen, R. D. & Herschman, H. R. *Eur. J. Biochem.* **118**, 527-531 (1981).
8. Karin, M. *et al. Nature* **308**, 513-519 (1984).
9. Sood, A. K., Pereira, D. & Weissman, S. M. *Proc. natn. Acad. Sci. U.S.A.* **78**, 616-620 (1981).
10. Gunning, P. *et al. Molec. cell. Biol.* **3**, 787-795 (1983).
11. Ringold, G. M., Dieckmann, B., Vannice, J. L., Trahey, M. & McCormick, F. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3964-3968 (1984).

Experiments are now under way to test directly the role of the consensus sequence in transcriptional regulation by interferon. If this sequence is involved, it may be long enough to be used to isolate genes directly from phage or cosmid libraries by hybridization, and would help in locating their regulatory regions. Both consensus and naturally occurring sequences might then be used to isolate the proteins responsible for interferon transcriptional regulation.

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12. Yoshie, O. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 649-653 (1984).
13. Vannice, J. L., Taylor, J. M. & Ringold, G. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4241-4245 (1984).
14. Richards, R. I., Heguy, A. & Karin, M. *Cell* **37**, 263-272 (1984).
15. Strachan, T., Sodoyer, R., Damotte, M. & Jordan, B. R. *EMBO J.* **3**, 887-894 (1984).
16. Malissen, M., Malissen, B. & Jordan, B. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 893-897 (1982).
17. Das, H. K., Lawrence, S. K. & Weissman, S. M. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3543-3547 (1983).
18. Bienz, M. & Pelham, H. R. B. *EMBO J.* **1**, 1583-1588 (1982).
19. Pelham, H. R. B. *Cell* **30**, 517-528 (1982).
20. Pelham, H. R. B. & Bienz, M. *EMBO J.* **1**, 1473-1477 (1982).
21. Donahue, T. F., Daves, R. S., Lucchini, G. & Fink, G. R. *Cell* **32**, 89-98 (1983).
22. Lucchini, G., Hinnebusch, A. G., Chen, C. & Fink, G. R. *Molec. cell. Biol.* **4**, 1326-1333 (1984).
23. Allen, G., Fantes, K. H., Burke, D. C. & Morser, J. *J. gen. Virol.* **63**, 207-212 (1982).
24. Karin, M. & Richards, R. I. *Nature* **299**, 797-802 (1982).
25. Wilbur, W. J. & Lipman, D. J. *Proc. natn. Acad. Sci. U.S.A.* **80**, 726-730 (1983).

Transient reversion of *ras* oncogene-induced cell transformation by antibodies specific for amino acid 12 of *ras* protein

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The proteins encoded by the *ras* oncogene are thought to trigger expression of the transformed phenotype in some types of cancer cells. In human cells, the *ras* protein family consists of several members including normal (proto-oncogene) and mutant (oncogene) forms¹⁻³. In general, the proto-oncogene forms are thought to be involved in the normal growth control of cells, while the mutant forms (which apparently result from somatic mutation of the normal *ras* genes) appear to be responsible, in part, for the loss of normal growth control. On microinjection into living normal cells, the purified *ras* oncogene protein (p21) induces a characteristic loss of growth control in cells within several hours^{4,5}. The mutant forms of the different *ras* proteins typically contain a single amino-acid change, usually at position 12 or less frequently at position 61 (ref. 6). Here we report that microinjection of antibodies specific for amino acid 12 of the oncogenic v-Ki-ras protein into cells transformed by this protein causes a transient reversion of the cells to a normal phenotype. The fact that this antibody inhibits binding of GTP to the v-Ki-ras protein supports the notion that GTP binding is essential to the transforming function of this oncogene product.

The role of *ras* proteins in cellular metabolism is not yet understood, but it is known that they are localized on the inner surface of the plasma membrane and bind GTP and GDP^{7,8}. Recent analysis of the normal and mutant forms of the human Ha-ras proteins purified from bacterial expression systems^{9,10} or present in the bacterial lysates after expression¹¹ has shown that *ras* proteins are able to hydrolyse GTP to GDP. It has been

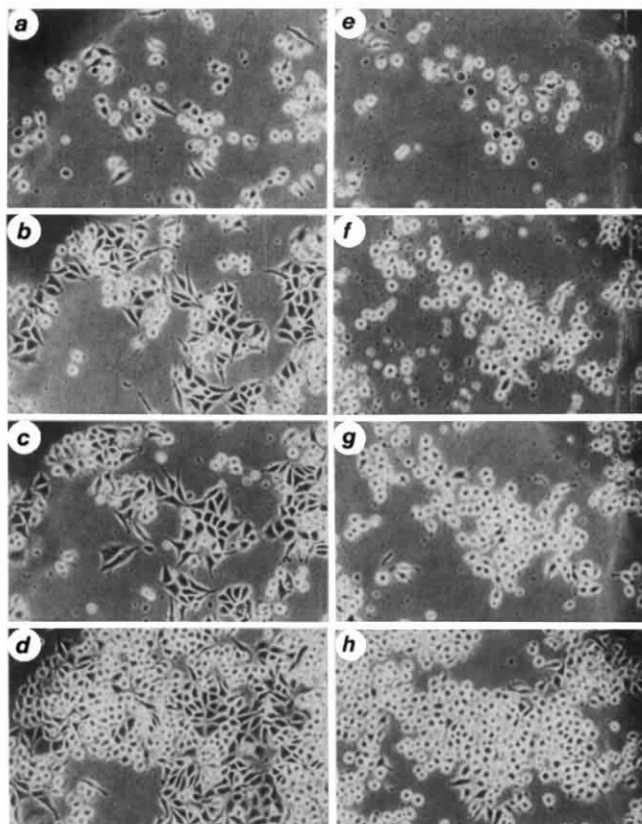


Fig. 1 Morphology of Ki-NRK cells after microinjection of anti-p21-Ser and control IgG. Approximately 35 Ki-NRK cells (growing in 10% fetal calf serum in Dulbecco's modified Eagle's medium) in each of the two test areas (marked by ink circles under the dish) were microinjected with anti-p21-Ser (a-d) or non-immune IgG (e-h) as described elsewhere¹⁸. Phase-contrast photomicrographs were taken of the two areas 30 min (a, e), 24 h (b, f), 36 h (c, g) and 48 h (d, h) after injection. Note that the cells injected with anti-p21-Ser show a normal flattened morphology for some time and grow at a slower rate than either the surrounding uninjected cells or the cells injected with the control IgG. $\times 4$.

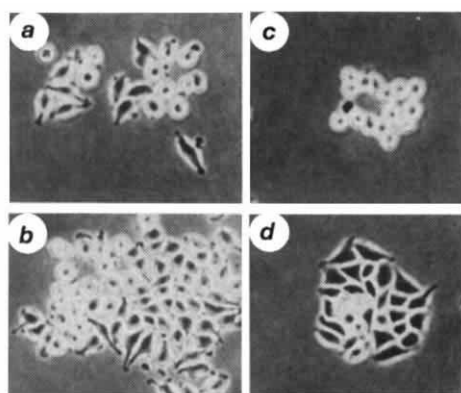


Fig. 2 Slower growth rate of Ki-NRK cells after microinjection of anti-p21-Ser antibody. Two areas of sparsely plated Ki-NRK cells growing on a dish (see Fig. 1 legend) were injected with either control IgG (a) or anti-p21-Ser IgG (c). The two areas were photographed immediately after injection (a and c) and 24 h later (b and d, respectively). Note the different rates of growth between the two areas of cells.

suggested that *ras* proteins function in a manner analogous to the 'G' regulatory protein of the adenylate cyclase system^{4,9-13}, which is also localized in the plasma membrane; this model is particularly attractive because G proteins activate adenylate cyclase through a GTP-dependent reaction, and inactivate the enzyme through hydrolysis of bound GTP to GDP and P_i . Position 12 mutants of human Ha-*ras* protein show a 10-fold reduction in rate of GTP hydrolysis⁹⁻¹¹, and may therefore represent abnormally activated regulatory proteins.

To obtain a biochemical probe capable of distinguishing between the normal and oncogenic forms of the *ras* proteins, antibodies were raised against *ras* (p21)-related peptides that differed at position 12. One antibody, designated anti-p21-Ser, was affinity-purified from the serum of rabbits immunized with the peptide ⁶Lys-Leu-Val-Val-Gly-Ala-Ser-Gly-Val-Gly-Lys Cys-¹⁸Ser (Ser at position 12) coupled covalently to carrier protein (R.C., G.W., D. Nitecki and F. McC., unpublished). Anti-p21-Ser was found to bind the v-Ki-*ras* protein (serine at position 12) but neither the v-Ha-*ras* protein (arginine at position 12) nor the *ras* protein containing glycine (normal) at position 12. In addition, biochemical studies showed that this

antibody blocked both the GTP-dependent autophosphorylation and GTP-binding activities of the v-Ki-*ras* protein, suggesting that the antibody and GTP bind to the same region of the protein.

Given this high degree of specificity of the affinity-purified antibody preparation for the oncogenic v-Ki-*ras* protein, as well as the ability of the antibody to inhibit the GTP binding activity of the v-Ki-*ras* protein, it seemed possible that introduction of this antibody preparation into living, transformed cells might block the biochemical action of the *ras* oncogene protein *in vivo*, without affecting the function of the proto-oncogene product. Using microinjection procedures, $\sim 5 \times 10^5$ molecules of purified IgG (either anti-p21-Ser or non-immune serum) were injected into the cytoplasm of NRK cells transformed by the v-Ki-*ras* oncogene (called Ki-NRK cells); this represents a 5-10-fold excess of antibody over the estimated amount of *ras* protein in these cells⁴. Within 15 h and persisting for 48 h after injection of the anti-p21-Ser antibodies, the Ki-NRK cells showed a flattened, normal morphology (Fig. 1a-d); after 48 h, the cells reverted to their rounded morphology. In contrast, cells on the same dish which had been injected with control IgG showed no obvious change in morphology (Fig. 1e-h). (Control IgGs included goat anti-mouse IgG and rabbit non-immune IgG.) Inspection of the photographs taken for these experiments indicated that in addition to the reversion of the transformed phenotype, the flattened cells appeared to grow at a slower rate than either cells injected with control IgG or surrounding uninjected cells; this is evident from Fig. 2, where cells injected with control IgG have tripled in number over 24 h post-injection while cells injected with the anti-p21-Ser IgG have only doubled in number over the same time period. Based on data from the genetic analysis of the yeast *ras* genes which indicated that complete elimination of *ras* gene expression yielded non-viable cells^{14,15}, we might predict that antibodies that block the function of both the normal and oncogenic forms of the *ras* proteins would kill or prevent growth of microinjected transformed mammalian cells. Figures 1 and 2 show, however, that the cells injected with anti-p21-Ser are indeed able to grow, a result in agreement with biochemical experiments which showed that this antibody binds only to the oncogenic form of the *ras* protein with serine at position 12 (R.C., G.W., N.A., D. Nitecki and F. McC., unpublished).

By using immunofluorescence microscopy, we were able to determine exactly which cells had been injected with the two antibody preparations. Those cells that had been injected with

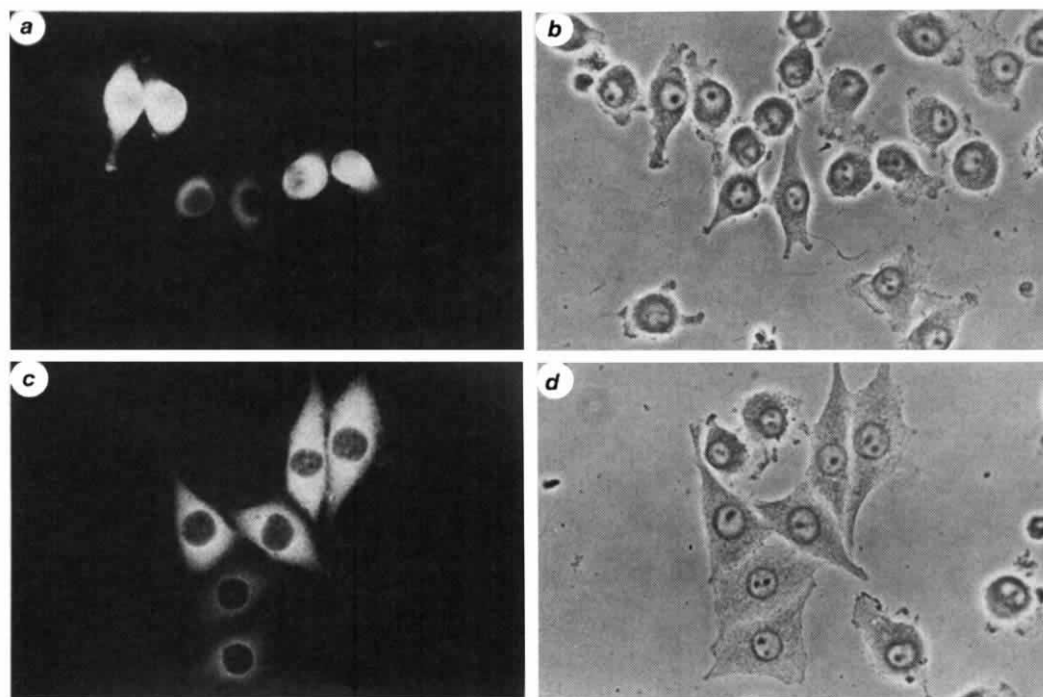


Fig. 3 Correspondence of the flattened appearance of Ki-NRK cells with microinjection of anti-p21-Ser antibodies. Ki-NRK cells (growing on glass coverslips) were injected with non-immune IgG (a, b) or anti-p21-Ser (c, d) and fixed (absolute methanol, -20°C , 5 min) 24 h after injection. The cells were treated with fluorescein isothiocyanate-labelled goat anti-rabbit IgG (Cappel Laboratories), which stained only the injected cells. The fields of cells were photographed by epifluorescence (a, c) and phase-contrast (b, d) microscopy with a Zeiss Photomicroscope III as described elsewhere¹⁸. a, b Represent the same field of cells injected with control IgG and c and d the same field of cells injected with anti-p21-Ser.

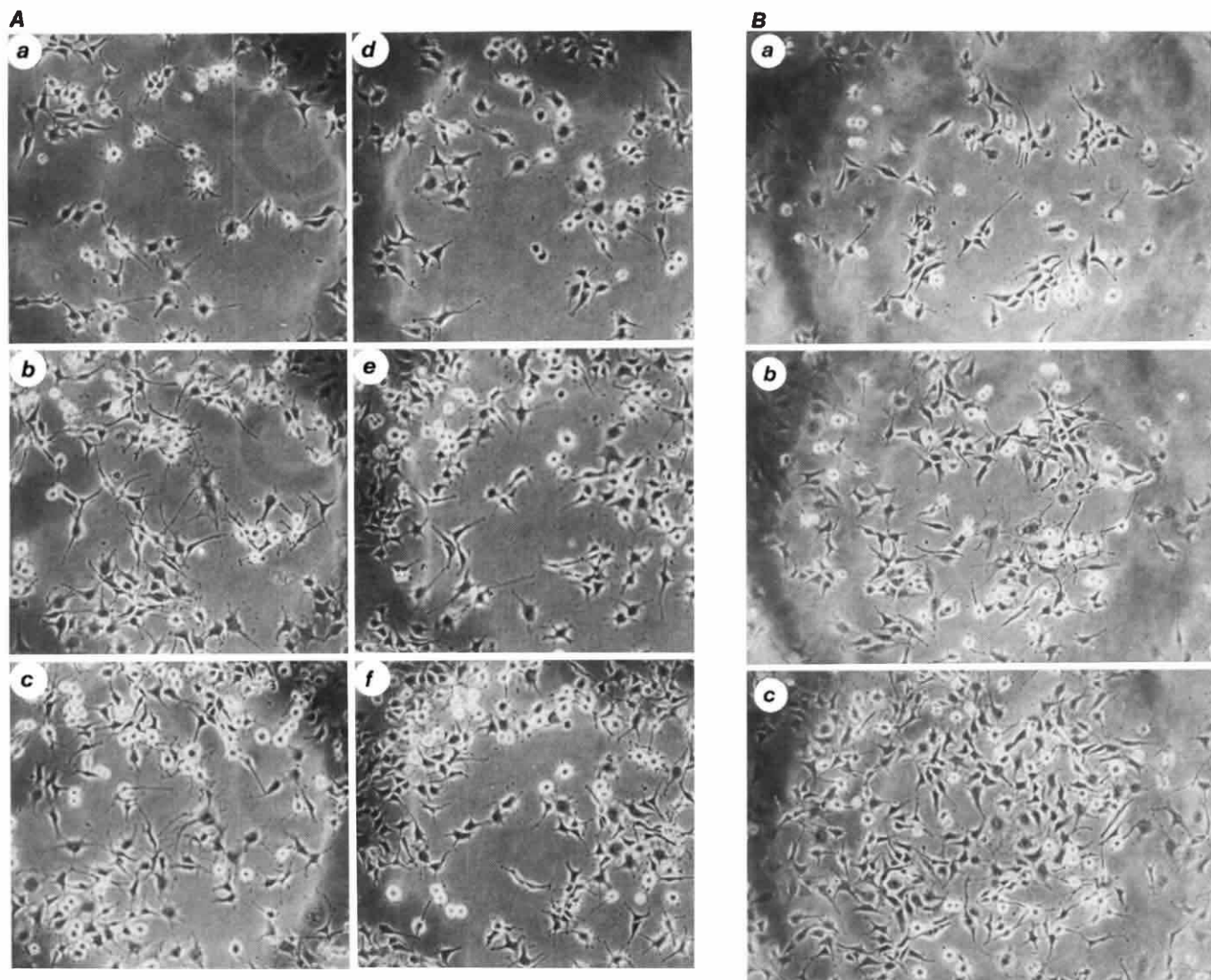


Fig. 4 Morphology of Ha-NRK cells after microinjection of: **A**, anti-p21-Ser and control IgG; and **B**, monoclonal antibody Y-238. **A**, Approximately 25 Ha-NRK cells in defined areas (marked by ink circles on the bottom of the dish) were injected with anti-p21-Ser (**a-c**) or non-immune IgG (**d-f**). The areas were photographed 30 min (**a, b**), 24 h (**c, d**) and 36 h (**e, f**) post-injection. No change in morphology or growth rate was observed for either antibody. **B**, Approximately 30 Ha-NRK cells were injected with monoclonal antibody Y-238 (ref. 16) and photographed 30 min (**a**), 24 h (**b**), and 36 h (**c**) after injection. Note the lack of effect of this monoclonal antibody on either the morphology or the growth rate of the cells.

anti-p21-Ser had the morphologically normal, flattened appearance (Fig. 3c, d) whereas cells injected with control IgG retained their rounded, transformed appearance (Fig. 3a, b).

To determine the specificity of the *in vivo* effect of the anti-p21-Ser preparation in terms of cells transformed by other oncogenic *ras* proteins, the anti-p21-Ser and control IgGs were injected (5×10^5 molecules per cell) into Ha-NRK cells (that is, NRK cells transformed by the v-Ha-*ras* oncogene protein, which has an arginine at position 12). Photomicroscopy indicated that neither antibody had an effect on these cells (Fig. 4A); this result is consistent with the observation that, *in vitro*, the anti-p21-Ser preparation does not react with the v-Ha-*ras* protein (R.C., G.W., N.A., D. Nitecki and F.McC., unpublished).

To determine whether the ability of anti-p21-Ser to induce reversion to normal phenotype is a general property of anti-p21 antibodies, one other antibody was tested by this assay. Further and co-workers have produced several rat-derived monoclonal antibodies against the v-Ha-*ras* protein¹⁶, including one that is relatively specific for the Ha-*ras* proteins (called Y-238); this antibody does not block GTP binding activity, does not discriminate between normal and mutant forms of the *ras* protein and probably binds to a site in the Ha-*ras* protein that is not influenced by amino acid 12 of the protein. Y-238 was microinjected

into Ha-NRK cells (1×10^6 molecules per cell), and the cells were examined by photomicroscopy for 48 h. Neither the Y-238 monoclonal antibody nor a control IgG preparation had any effect on the morphology or growth rate of the cells (Fig. 4B); similar results were obtained using Y-259 anti-p21 antibody. Thus, the antibody either binds to a non-essential site on the *ras* protein or does not bind at all to the *ras* protein in these cells.

These results, together with the ability of anti-p21-Ser to block GTP binding by p21 *in vitro* (R.C., G.W., N.A., D. Nitecki and F.McC., unpublished), are consistent with the idea that the interaction of p21 with GTP is crucial to the transforming function of the *ras* gene. However, further studies are required to examine the possibility that the antibody disrupts other important activities of p21 such as membrane localization or interactions with cellular proteins. Whatever mechanism is responsible for the observed effects of the microinjected anti-p21-Ser on cell behaviour, the data presented here indicate that antibodies capable of inactivating oncogene but not proto-oncogene products can block the expression of the transformed phenotype in living cells. Gallick *et al.*, using antibodies directed against the p37^{mos} oncogene product of the Moloney murine sarcoma virus, reached a similar conclusion; microinjection of the antibodies yielded at least a different morphology in transformed cells¹⁷.

When antibodies specific for other single mutations in the *ras* protein family (N.A. *et al.*, in preparation) become available, we will be able to extend this approach to inhibition of the function of the other forms of the oncogenic *ras* proteins in living cell and biochemical systems. Such experiments, coupled with studies in which the human *ras* oncogene protein is injected into normal cells, resulting in transient proliferation^{4,5}, provide new ways of examining the physiology and cell biochemistry of the transformation process.

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- Der, C. J., Krontiris, T. G. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3637-3640 (1982).
- Parada, L. F., Tabin, C. J., Shih, C. & Weinberg, R. A. *Nature* **297**, 474-478 (1982).
- Shimizu, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 2112-2116 (1983).
- Feramisco, J. R., Kamata, T., Gross, M., Rosenberg, M. & Sweet, R. W. *Cell* **38**, 109-117 (1984).
- Stacey, D. & Kung, H. *Nature* **310**, 508-511 (1984).
- Marshall, C. J. & Rigby, P. W. J. *Cancer Surv.* **3**, 183-214 (1984).
- Willingham, M. C., Partan, L., Shih, T. Y. & Scolnick, E. M. *Cell* **19**, 1005-1014 (1980).
- Shih, T. Y., Papageorge, A. G., Stoker, P. E., Weeks, M. O. & Scolnick, E. M. *Nature* **287**, 686-691 (1980).
- Sweet, R. W. *et al. Nature* **311**, 273-275 (1984).
- Gibbs, J. B., Sigal, I. S., Poe, M. & Scolnick, E. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5704-5708 (1984).
- McGrath, J. P., Capon, D. J., Goeddel, D. V. & Levinson, A. D. *Nature* **310**, 644-649 (1984).
- Gilman, A. G. *Cell* **36**, 577-579 (1984).
- Kamata, T. & Feramisco, J. R. *Cancer Cells* **1**, 11-16 (1984).
- Tatchell, K., Chaleff, D., Defeo-Jones, D. & Scolnick, E. *Nature* **309**, 523-527 (1984).
- Kataoka, T. *et al. Cell* **37**, 437-455 (1984).
- Furth, M. E., Davis, L. J., Fleurdelys, B. & Scolnick, E. M. *J. Virol.* **43**, 294-304 (1982).
- Gallick, G. E., Brown, D. B., Murphy, E. C. Jr & Arlinghaus, R. B. in *Modern Approaches to Vaccines* (eds Chanock, R. M. & Lerner, R. A.) 407-413 (Cold Spring Harbor Laboratory, New York).
- Wang, K., Feramisco, J. R. & Ash, J. F. *Meth. Enzym.* **85**, 514-562 (1982).

Production of 'hybrid' antibiotics by genetic engineering

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The recent development of molecular cloning systems in *Streptomyces*¹⁻⁴ has made possible the isolation of biosynthetic genes for some of the many antibiotics produced by members of this important genus of bacteria⁵⁻¹⁰. Such clones can now be used to test the idea that novel antibiotics could arise through the transfer of biosynthetic genes between streptomycetes producing different antibiotics¹¹. The likelihood of a 'hybrid' compound being produced must depend on the substrate specificities of the biosynthetic enzymes, about which little is known. In attempts to demonstrate hybrid antibiotic production, we therefore began with strains producing different members of the same chemical class of compounds in order to maximize the chance of success. Here we report the production of novel compounds by gene transfer between strains producing the isochromanonequinone antibiotics actinorhodin¹²,

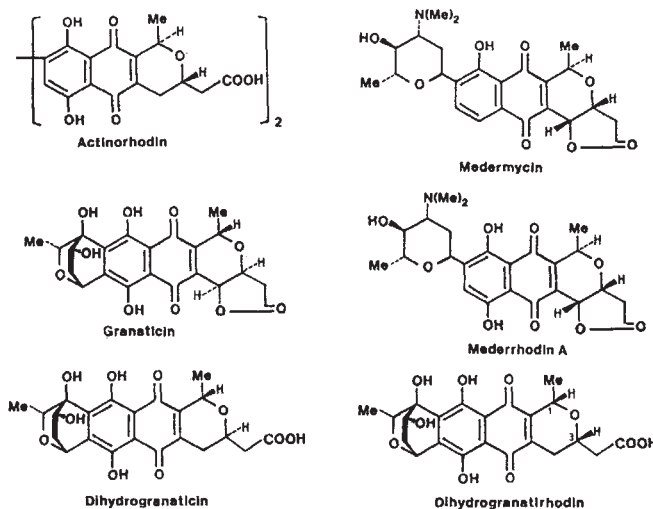


Fig. 1 Structures of the isochromanonequinone antibiotics. The absolute stereochemistry of dihydrogranaticirhodin is tentative (see text).

granaticin¹³ and medermycin¹⁴. These experiments were made possible by the recent cloning of the whole set of genes for the biosynthetic pathway of actinorhodin from *Streptomyces coelicolor* A3(2) (ref. 8). We believe that this represents the first report of the production of hybrid antibiotics by genetic engineering.

Plasmid pIJ2303 (ref. 8) consists of the low-copy-number vector plasmid pIJ922, derived from SCP2* (ref. 15), with 32.5 kilobases (kb) of *S. coelicolor* A3(2) DNA inserted at its unique *Bam*HI site⁸. That this inserted DNA carries the complete cluster of genes (*act*) for the biosynthesis of actinorhodin (Fig. 1) from simple primary metabolites (probably acetate) is shown by complementation of all available classes of *S. coelicolor act* mutants by pIJ2303 and by the production of actinorhodin by several species of *Streptomyces* (including *S. parvulus* ATCC 12434, *S. peucetius* ATCC 29050, *S. tanashiensis* KA-415 and *S. glaucescens* ETH 22794) carrying pIJ2303 (F.M., H.M.K. and D.A.H., unpublished).

In an analysis of the organization and expression of the *act* genes, segments of the total *act* DNA of pIJ2303 have recently been inserted (F.M., unpublished) into the *Bam*HI site of pIJ922 or into the *Bam*HI, *Bgl*II or *Pst*I sites of pIJ940 (a second low-copy-number vector derived from SCP2*; ref. 15). Some of these subclones, carrying different transcription units of the *act* region, were introduced by transformation into *Streptomyces* sp. AM-7161 (the producer of medermycin), *Streptomyces violaceoruber* Tü22 (the producer of granaticin), and a mutant (B1140) of Tü22 blocked in granaticin synthesis, by selecting for the thiostrepton-resistance marker of the vectors. The efficiency of transformation of these cultures, using standard conditions developed for *Streptomyces lividans* and *S. coelicolor*¹⁶, by DNA of the clones (isolated from *S. coelicolor* A3(2) derivatives) was only 1-10 per µg of plasmid DNA, compared with 10⁶-10⁷ in *S. coelicolor* A3(2) (ref. 8). These reduced frequencies may reflect the operation of restriction systems, as well as the fact that no attempt was made to optimize transformation conditions for these strains. Nevertheless, transformants were obtained in all of the combinations attempted (Table 1).

Actinorhodin, granaticin and medermycin are all acid-base indicators which confer characteristic colours on the cultures producing them: red ↔ blue for actinorhodin; red ↔ purple for granaticin; and yellow ↔ brown for medermycin. It was apparent from the colours of AM-7161 and B1140 carrying pIJ2303 that the *S. coelicolor act* genes were being expressed in the recipient strains, as blue pigments were present in alkaline conditions. More strikingly, the colours of the cultures produced by introducing certain of the partial *act* clones into the medermycin producer (AM-7161) indicated that one or more novel compounds were present. Notable was the bright purple colour, at

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Table 1 Antibiotics produced by the *Streptomyces* strains

Strain	Colour of culture†		Relevant compounds identified‡
	Acidic	Alkaline	
<i>S. coelicolor</i> A3(2)	Red	Blue	Actinorhodin
<i>Streptomyces</i> sp. AM-7161	Yellow	Brown	Medermycin
AM-7161/pIJ2303*	Red	Blue	Medermycin, actinorhodin
AM-7161/pIJ2301*	Red	Purple	Mederrhodin A, medermycin
AM-7161/pIJ2304	Yellow	Brown	Medermycin
AM-7161/pIJ2308	Yellow	Brown	NT
AM-7161/pIJ2312	Yellow	Brown	NT
AM-7161/pIJ2315*	Red	Purple	Mederrhodin A, mederrhodin B, medermycin
AM-7161/pIJ2316*	Red	Purple	Mederrhodin A, medermycin
AM-7161/pIJ2317	Yellow	Brown	NT
<i>S. violaceoruber</i> Tü22	Red	Blue-purple	Granaticin, dihydrogranaticin
Tü22/pIJ2303*	Red	Blue-purple	Dihydrogranatirhodin, actinorhodin
<i>S. violaceoruber</i> B1140	Yellow	Brown	NT
B1140/pIJ2303	Red	Blue-purple	Granaticin, dihydrogranaticin, actinorhodin
B1140/pIJ2301	Yellow	Brown	NT
B1140/pIJ2302	Yellow	Brown	NT
B1140/pIJ2304	Yellow	Brown	NT
B1140/pIJ2308*	Red	Blue-purple	Granaticin, dihydrogranaticin
B1140/pIJ2310	Yellow	Brown	NT
B1140/pIJ2311	Yellow	Brown	NT
B1140/pIJ2314	Yellow	Brown	NT

* Plasmid DNA from these cultures was shown to be indistinguishable in structure from the same plasmid originally isolated in *S. coelicolor*, as judged by *Bam*HI digestion.

† To characterize the pigments present in AM-7161 carrying various clones, cultures were grown in liquid medium (seed stage: 2% glucose, 0.3% peptone, 0.3% beef extract, 0.1% yeast extract, 0.3% NaCl, 0.1% CaCO₃, pH 7.0, for 48 h at 28 °C; production stage: 1.5% glycerol, 1% soybean meal, 0.3% NaCl, pH 7.0, with shaking for 96 h at 28 °C, after addition of a 10% inoculum from the seed stage). The broth was adjusted to pH 7.0 with 1 M HCl and extracted with chloroform. After concentration of the extracts, they were analysed by silica-gel TLC run in chloroform/methanol (5:1). The pigments produced by cultures of Tü22 carrying pIJ2303, and B1140 carrying pIJ2303 and pIJ2308, were obtained from fermentations in a medium containing 1% raw ground peanuts, 1% glucose, 0.5% NaCl, 0.35% CaCO₃, 70 mg l⁻¹ histidine, 10 mg l⁻¹ guanine, 15 mg l⁻¹ thiostrepton in baffled flasks shaken at 300 r.p.m. at 28 °C. Seed cultures were grown for 24 h and production cultures for 48 h (granaticin-related pigments) or 144 h (actinorhodin) after addition of 5% inoculum. The broth was then adjusted to pH 2.0 and the culture was filtered. The residue was resuspended in 1 M NaOH and re-filtered. After adjusting the pH of the filtrate to 2.0, the precipitated crude pigments were collected by centrifugation and dried under vacuum. TLC analyses were done on oxalic acid-impregnated silica gel (chloroform/ethyl acetate, 6:4).

‡ Cultures were grown on R2YE plates¹⁶ (containing histidine and guanine in the case of B1140, which carries two auxotrophic mutations); after 4–5 days they were exposed to the fumes of either concentrated HCl or ammonia solution. NT, not tested.

alkaline pH, of the culture carrying pIJ2315; cultures carrying pIJ2301 and pIJ2316 had a duller purple colour. B1140 carrying pIJ2308 had a colour resembling that of granaticin, and this result also demonstrated a 'cooperation' between gene products in the actinorhodin and granaticin pathways.

We characterized the pigments present in AM-7161 carrying various clones. The results (Table 1) confirmed that AM-7161 carrying pIJ2315 produced, in addition to medermycin, large amounts of a novel compound; this material was also present, in smaller quantities, in AM-7161 carrying pIJ2301 and pIJ2316, and was termed 'mederrhodin A'. A second novel compound ('mederrhodin B') was present in the aqueous phase after chloroform extraction of the AM-7161/pIJ2315 broth. Interestingly, AM-7161 carrying pIJ2303 produced abundant actinorhodin, together with medermycin, but no novel compounds were detected.

Mederrhodin A was purified by passing the acidified broth of AM-7161/pIJ2315 through a Diaion HP-20 column (Mitsubishi) to adsorb medermycin and related compounds. The column was washed with deionized water and the pigments were eluted with 70% aqueous acetone. After evaporation of the acetone from appropriate fractions, the concentrate was adjusted to pH 7.0 with 1 M NaOH and extracted twice with equal volumes of chloroform. Mederrhodin A was then purified by preparative TLC. Structural determination of field desorption-mass spectroscopy and element analysis revealed hydroxymedermycin, and the position of the hydroxyl group was determined by ultraviolet and ¹H-NMR spectroscopy (Fig. 1). Mederrhodin A, like medermycin, was found to have antibiotic activity against various Gram-positive bacteria.

The pigments produced by cultures of Tü22 carrying pIJ2303, and B1140 carrying pIJ2303 and pIJ2308, were obtained as described in Table 1. B1140/pIJ2303 and B1140/pIJ2308 pro-

duced a mixture of granaticin and dihydrogranaticin (Fig. 1), pigments characteristic of the Tü22 strain; actinorhodin was also present in B1140/pIJ2303, but no novel compounds were detected. However, Tü22 carrying pIJ2303 produced, in addition to actinorhodin, a novel compound related to dihydrogranaticin; this compound was named 'dihydrogranatirhodin'. Interestingly, very little granaticin or dihydrogranaticin was present.

Dihydrogranatirhodin was purified by column chromatography on oxalic acid-treated silica gel (chloroform) and preparative TLC, and was converted to the methyl ester as described for dihydrogranaticin¹⁷. The mass spectrum gave the molecular formula C₂₃H₂₄O₁₀ and the ultraviolet/visible spectrum indicated the presence of the same 5,8-dihydroxy-1,4-naphthoquinone chromophore as in dihydrogranaticin. The ¹H-NMR spectrum closely resembles that of dihydrogranaticin, but shows up-field shifts of the signals for H-3 and H-1, as has been seen in synthetic model compounds with *cis*-arrangement of these two hydrogens¹⁷. This was confirmed by observation of a nuclear Overhauser enhancement of 5.5% for H-3 on irradiation of H-1. Hence, dihydrogranatirhodin is either the C-3 or the C-1 epimer of dihydrogranaticin. As the configuration at C-3 in actinorhodin is determined early in the biosynthesis by reduction of the C-3 carbonyl group (X.G.He, S. P. Cole and H.G.F., unpublished), we suggest that dihydrogranatirhodin has the actinorhodin (3S) configuration at C-3 and the granaticin (1S) configuration at C-1 (Fig. 1).

The demonstration of novel compounds in hybrid strains prepared by transferring actinorhodin genes into both the granaticin- and the medermycin-producer augurs well for the widespread usefulness of genetic engineering as a new tool in antibiotic discovery. There seems little doubt that dihydrogranatirhodin and the mederrhodins are indeed the products of 'hybrid' biosynthetic pathways arising through an interaction

between enzymes encoded by structural genes originating from two different streptomycetes, rather than the results of the activation of latent genetic information in the recipient strains. (The latter mechanism is the most probable explanation for the three reports of the discovery of novel compounds through natural inter-strain matings (refs 18–20; discussed in ref. 21).) The evidence is most compelling for mederrhodin A, since, of the clones tested, pIJ2301, pIJ2315 and pIJ2316 (which led to mederrhodin A synthesis by AM-7161) all contain a complete transcription unit (absent from pIJ2304, pIJ2308, pIJ2312 and pIJ2317) which complements class V *act* mutants of *S. coelicolor* (F.M., unpublished); such mutants have recently been shown to be blocked in the corresponding hydroxylation involved in actinorhodin biosynthesis (S. P. Cole and H.G.F., unpublished). A true metabolic cooperation between the actinorhodin and granaticin biosynthetic enzymes is indicated also by complementation of the B1140 mutant by pIJ2308, but not by the other clones tested. B1140 can act as a secretor in co-synthesis tests with *S. coelicolor act* mutants of classes I, III and VII, but not of classes IV, V and VI²², suggesting that its block is equivalent to that of class IV *act* mutants. Significantly, pIJ2308 is the only clone tested that carries a complete transcription unit for the class IV *act* gene (F.M., unpublished). Moreover, the 'mixed' stereochemistry found in dihydrogranatirhodin has not previously been encountered in any isochromanquinone antibiotic, even though a strain of *Streptomyces roseofulvus* has recently been found to produce both frenolicin B (with the same configuration of the pyranoid ring as actinorhodin) and nanaomycins A and β A [with the same configuration as granaticin (S.O. *et al.*, unpublished)].

The different results obtained with AM-7161 and Tü22 carrying the complete set of actinorhodin biosynthetic genes is interesting. In AM-7161, the donor and recipient gene sets appear to operate independently, with production of the two parental antibiotics but no novel compounds. Only when a partial *act* clone was introduced into the medermycin-producer was a hybrid product formed. In contrast, Tü22 carrying pIJ2303 produced dihydrogranatirhodin (as well as some actinorhodin), to the almost total exclusion of the normal antibiotics of the recipient. This differing behaviour may reflect several factors, including the relative specificities and affinities of the biosynthetic enzymes for their substrates, the regulation of gene expression and any possible 'channelling' of precursors within individual biosynthetic pathways. Such considerations must be borne in mind in the future use of genetic engineering in antibiotic discovery, or in attempts to modify existing antibiotics in predetermined ways.

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1. Bibb, M. J., Schottel, J. L. & Cohen, S. N. *Nature* **284**, 526–531 (1980).
2. Suarez, J. E. & Chater, K. F. *Nature* **286**, 527–529 (1980).
3. Thompson, C. J., Ward, J. M. & Hopwood, D. A. *Nature* **286**, 525–527 (1980).
4. Bibb, M. J., Chater, K. F. & Hopwood, D. A. in *Experimental Manipulation of Gene Expression* (ed. Inouye, M.) 53–82 (Academic, New York, 1983).
5. Feitelson, J. S. & Hopwood, D. A. *Molec. gen. Genet.* **190**, 394–398 (1983).
6. Gil, J. A. & Hopwood, D. A. *Gene* **25**, 119–132 (1983).
7. Chater, K. F. & Bruton, C. J. *Gene* **26**, 67–78 (1983).
8. Malpartida, F. & Hopwood, D. A. *Nature* **309**, 462–464 (1984).
9. Bailey, C. R., Butler, M. J., Normansell, N. D., Rowlands, R. T. & Winstanley, D. J. *Bio/Technology* **2**, 808–811 (1984).
10. Jones, G. H. & Hopwood, D. A. *J. biol. Chem.* **259**, 14151–14157 (1984).
11. Hopwood, D. A. in *Beta-lactam Antibiotics* (eds Salton, M. R. J. & Shockmann, G. D.) 585–598 (Academic, New York, 1981).
12. Brockmann, H., Zeeck, A., van der Merve, K. & Müller, W. *Justus Liebig Annl. Chem.* **698**, 3575–3579 (1966).
13. Carbaz, R. *et al. Helv. chim. Acta* **40**, 1262–1269 (1957).
14. Ogura, H. & Furuhata, K. *Abstr. 9th Int. Congr. Heterocyclic Chem.*, 114 (1983).
15. Lydiate, D. J., Malpartida, F. & Hopwood, D. A. *Gene* (in the press).
16. Thompson, C. J., Ward, J. M. & Hopwood, D. A. *J. Bact.* **151**, 668–677 (1982).
17. Pyrek, J. S.-P., Achmatowicz, O. & Zamojski, A. *Tetrahedron* **33**, 673–680 (1977).
18. Ihn, W., Schlegel, B., Fleck, W. F. & Sedmera, P. *J. Antibiot., Tokyo* **33**, 1457–1461 (1980).
19. Mazieres, N., Peyré, M. & Pénasse, L. *J. Antibiot., Tokyo* **34**, 544–550 (1981).
20. Traxler, P., Schupp, T., Fuhrer, H. & Richter, W. *J. Antibiot., Tokyo* **34**, 971–979 (1981).
21. Hopwood, D. A. in *Biochemistry and Genetic Regulation of Commercially Important Antibiotics* (ed. Vining, L. C.) 1–23 (Addison-Wesley, Reading, Massachusetts, 1983).
22. Rudd, B. A. M. & Hopwood, D. A. *J. gen. Microbiol.* **114**, 35–43 (1979).

Molecular cloning of Ancient Egyptian mummy DNA

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Artificial mummification was practised in Egypt from ~2600 BC until the fourth century AD. Because of the dry Egyptian climate, however, there are also many natural mummies preserved from earlier as well as later times. To elucidate whether this unique source of ancient human remains can be used for molecular genetic analyses, 23 mummies were investigated for DNA content. One 2,400-yr-old mummy of a child was found to contain DNA that could be molecularly cloned in a plasmid vector. I report here that one such clone contains two members of the *Alu* family of human repetitive DNA sequences, as detected by DNA hybridizations and nucleotide sequencing. These analyses show that substantial pieces of mummy DNA (3.4 kilobases) can be cloned and that the DNA fragments seem to contain little or no modifications introduced postmortem.

Samples were removed from 23 different mummies and mummy fragments. These specimens varied in age from the Sixth Dynasty (~2370–2160 BC) to late Roman times. Whenever possible, representative samples were taken from all the different tissues that could be identified visually. After rehydration and preparation of microscopical sections¹, the samples were studied using conventional histological stains as well as staining by ethidium bromide, which allows detection of small amounts of DNA. The cartilage cells from the outer ear of a mummified female head (Egyptian Museum, Berlin, GDR) as well as cells in the epidermis and subcutaneous tissues of a male head (Viktoria Museum, Uppsala; inventory number VM3251) proved to contain identifiable cell nuclei stainable by ethidium bromide. When the DNA from the latter sample was extracted, however, it was found to contain modified pyrimidines^{2,3} and was intractable to molecular cloning. In contrast, cells of the epidermis and several subcutaneous structures from a less than 1-yr-old boy from the collections of the Egyptian Museum,

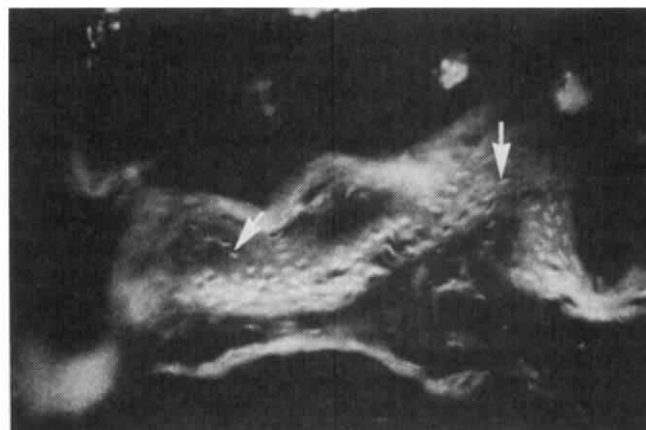
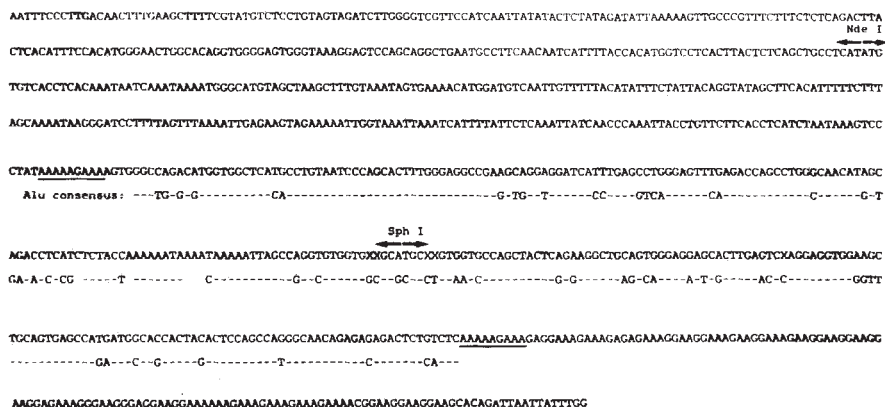


Fig. 1 Tissue section of skin from the left lower leg of the Berlin mummy used for molecular DNA cloning. Ethidium bromide staining allows the visualization of nucleic acids in the cell nuclei (arrows).

Methods. Small tissue samples were rehydrated in an aqueous solution of 1% Na₂CO₃ (w/v), 0.5% formalin and 28.5% ethanol¹ for 48 h. Paraffin embedding and sectioning were performed using routine protocols. After deparaffination, the sections were stained in a 5 µg ml⁻¹ solution of ethidium bromide in phosphate-buffered saline (PBS) for 30 min, followed by extensive washing in PBS and inspection under a Leitz fluorescence microscope. ×180.

These results establish the feasibility of faithfully cloning substantial pieces of genomic DNA from biological remains of great antiquity. The yield of DNA from this unusually well-preserved mummy was $\sim 20 \mu\text{g}$ per g dried tissue, which approaches 5% of the amount expected from fresh tissue. Note, however, that most of the mummy samples investigated seem to be devoid of nucleic acids. Interestingly, the general histological preservation of the various mummy tissues was much better in superficial tissues and peripheral parts of the bodies than in the more deeply situated tissues, an observation made also by others^{12,13}. This probably results from the fact that the mummifi-



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1. Sandison, A. T. *Stain Technol.* **30**, 277-283 (1955).
2. Pääbo, S. *Das Altertum* **30**, 213-218 (1984).
3. Pääbo, S. *J. archaeol. Sci.* (submitted).
4. Possnert, G. *Nucl. Instrum. Meth. Phys. Res. B5*, 159-161 (1984).
5. Graham, D. E. *Analyst. Biochem.* **85**, 609-613 (1978).
6. Vieira, J. & Messing, J. *Gene* **19**, 259-268 (1982).
7. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237-251 (1977).
8. Schmid, C. W. & Jelinek, W. *R. Science* **216**, 1065-1070 (1982).
9. Southern, E. M. *J. molec. Biol.* **98**, 504-517 (1975).
10. Deininger, P. L., Jolly, D. J., Rubin, C. M., Friedmann, T. & Schmid, C. W. *J. molec. Biol.* **151**, 17-33 (1981).
11. Barker, W. C. *et al. Nucleic Acid Sequence Database* Version 22, National Biomedical Research Foundation (Washington, DC, 1984).
12. Daniels, F. Jr & Post, P. W. *Adv. Biol. Skin* **10**, 279-292 (1970).
13. Chapel, T. A., Mehregan, A. H. & Reymann, T. A. *J. Am. Acad. Derm.* **4**, 27-30 (1981).
14. Lucas, A. J. *Egyptol. Archaeol.* **18**, 125-140 (1932).
15. Higuchi, R., Bowman, B., Freiberger, M., Ryder, O. A. & Wilson, A. C. *Nature* **312**, 282-284 (1984).
16. Trigger, B. G. *The Cambridge History of Africa* (eds Fage, J. D. & Oliver, R.) 478-547 (Cambridge University Press, 1982).
17. Harrison, R. G., Connolly, R. C. & Abdalla, A. *Nature* **224**, 325-326 (1969).
18. Reeves, C. N. *Göttinger Miszellen* **54**, 61-71 (1982).
19. Harris, J. E. *et al. Science* **200**, 1149-1151 (1978).
20. Gerger, J. P., Stern, R. H. & Wensink, P. C. *Nucleic Acids Res.* **7**, 2115-2136 (1979).
21. Larhammar, D., Servenius, B., Rask, L. & Peterson, P. A. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
22. Maxam, A. M. & Gilbert, W. *Meth. Enzymol.* **65**, 499-560 (1980).

Mathematics

Fast Transforms: Algorithms, Analyses, Applications. DOUGLAS F. ELLIOTT and K. RAMA-MOHAN RAO (eds). Academic: 1982. Pp. 488. ISBN 0-12-237080-5. \$70.00.

Handbook of Applicable Mathematics. Vol. VI, Part A: Statistics. EMLYN LLOYD (ed.). Wiley: 1984. Pp. 498. ISBN 0-471-90274-8. £88.

A Model-Management Framework for Mathematical Programming. By KENNETH H. PALMER *et al.* Wiley: 1984. Pp. 392. ISBN 0-471-80472-X. £42.00.

Multivariate Descriptive Statistical Analysis: Correspondence Analysis and Related Techniques for Large Matrices. By LUDOVIC LEBART, ALAIN MORINEAU and KENNETH M. WARWICK. Wiley: 1984. Pp. 231. ISBN 0-471-86743-8. £33.20.

Probability Theory and Mathematical Statistics for Engineers. By V.S. PUGACHEV. Translated by I.V. SINITSYNA. Pergamon: 1984. Pp. 450. ISBN 0-08-029148-1. £50, \$90.

Spectral Methods for Partial Differential Equations. ROBERT G. VOIGT, DAVID GOTTLIEB and M. YOUSUFF HUSSAINI (eds). Wiley: 1984. Pp. 267. ISBN 0-89871-195-9. £19.65.

The Universal Equation Solver. By NOEL KANTARIS and PATRICK F. HOWDEN. Wiley: 1983. Pp. 195. Pbk ISBN 0-905104-40-4. Pbk £6.50.

University of Strathclyde Seminars in Applied Mathematical Analysis: Multiparameter Problems. G.F. ROACH (ed.). Shiva Publishing, The Hawthorns, 64 Welsh Row, Nantwich, Cheshire CW5 5ES, UK: 1984. Pp. 101. Hbk ISBN 1-85014-000-6; pbk ISBN 1-85014-001-4. Hbk £12.50; pbk £8.50.

Space Sciences

Astrophysical Techniques. By G.R. KITCHIN. Adam Hilger: 1984. Pp. 438. Hbk ISBN 0-85274-461-7; pbk ISBN 0-85274-484-6. Hbk £40; pbk £15.

The Catastrophic Universe: An Essay in the Philosophy of Cosmology. By A.G. PACHOLCZYK. Pachart: 1984. Pp. 120. ISBN 0-88126-702-3. \$9.95.

Colours of the Stars. By DAVID MALIN and PAUL MURDIN. Cambridge University Press: 1984. Pp. 198. ISBN 0-521-25714-X. £13.95, \$27.95.

Cosmology of the Early Universe. L.Z. FANG and R. RUFFINI (eds). Wiley: 1984. Pp. 305. Hbk ISBN 9971-950-92-8; pbk ISBN 9971-950-93-6. Hbk £31.35.

From Falling Bodies to Radio Waves. By EMILIO SEGRE. W.H. Freeman: 1984. Pp. 298. Hbk ISBN 0-7167-1481-7; pbk ISBN 0-7167-1482-5. Hbk \$24.95, pbk \$13.95.

A Hundred Billion Stars. By MARIO RIGUTTI. MIT Press: 1984. Pp. 285. ISBN 0-262-18111-8. £22.50.

Searching Between the Stars. By LYMAN SPITZER Jr. Yale University Press: 1984. Pp. 179. Hbk ISBN 0-300-02709-5; pbk ISBN 0-300-03247-1. Pbk £7.95.

The Surface of Mars. By MICHAEL H. CARR. Yale University Press: 1984. Pp. 232. Pbk ISBN 0-300-03242-0. Pbk £19.95.

Physics

Applied Atomic Collision Physics, Vol. 4. Condensed Matter. SHELDON DATZ (ed.). Academic: 1983. Pp. 630. ISBN 0-12-478804-1. \$79.

Inert Gases: Potentials, Dynamics, and Energy Transfer in Doped Crystals. M.L. KLEIN (ed.). Springer-Verlag: 1984. Pp. 266. ISBN 3-540-13128-0/0-387-13128-0. DM 78, \$30.60.

Multiphoton Spectroscopy of Molecules. By S.H. LIN *et al.* Academic: 1984. Pp. 260. ISBN 0-12-450520-1. \$59, £41.50.

Nucleon-Nucleon Interaction and Nuclear Many-Body Problems. S.S. WU and T.T.S. KUO (eds). Wiley: 1984. Pp. 748. ISBN 9971-966-35-2. £63.70.

The Physics of Hydrogenated Amorphous Silicon I. Structure, Preparation, and Devices. Topics in Applied Physics, Vol. 55. J.D. JOANNOPOULOS and G. LUCOVSKY (eds). Springer-Verlag: 1984. Pp. 287. ISBN 3-540-12807-7/0-387-12807-7. DM 118, \$45.80.

The Physics of Hydrogenated Amorphous Silicon II. Topics in Applied Physics, Vol. 56. J.D. JOANNOPOULOS and G. LUCOVSKY (eds). Springer-Verlag: 1984. Pp. 360. Hbk ISBN 3-540-12808-5. Hbk DM118.00, \$44.00.

Proceedings of the Fourth Course of the International School of Intermediate Energy Nuclear Physics. R. BERGERE, S. COSTA and C. SCHAEFER (eds). Wiley: 1984. Pp. 460. ISBN 9971-950-22-7. £43.

Proceedings of the 1st Hellenic School on Elementary Particle Physics. T. PAPADOPOULOU and N.D. TRACAS (eds). Wiley: 1984. Pp. 689. ISBN 9971-950-99-5. £56.80.

The Quantum Theory of Unimolecular Reactions. By H.O. PRITCHARD. Cambridge University Press: 1984. Pp. 175. ISBN 0-521-25711-5. £25, \$49.50.

Rare Halos, Mirages, Anomalous Rainbows and Related Electromagnetic Phenomena. WILLIAM R. CORLISS (ed.). Sourcebook Project, PO Box 107, Glen Arm, Maryland 21057: 1984. Pp. 238. ISBN 0-915554-12-7. \$12.95.

Relativity, Cosmology, Topological Mass and Supergravity. C. ARAGONE (ed.). Wiley: 1984. Pp. 296. ISBN 9971-950-95-2. £37.25.

Theory of Jets in Electron-Positron Annihilation. Springer Tracts in Modern Physics, Vol. 102. By GUSTAV KRAMER. Springer-Verlag: 1984. Pp. 140. Hbk ISBN 3-540-13068-3. Hbk DM 74.00, \$29.00.

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VLSI Electronics: Microstructure Science. NORMAN G. EINSPRUCH and DALE M. BROWN (eds). Academic: 1984. Pp. 527. ISBN 0-12-234108-2. \$69, £48.50.

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Chemistry

Advances in Organometallic Chemistry, Vol. 22. F.G.A. STONE and ROBERT WEST (eds). Academic: 1983. Pp. 326. ISBN 0-12-031122-4. \$55.00.

The Alkaloids, Vol. 21. Chemistry & Pharmacology. ARNOLD BROSSI (ed.). Academic: 1983. Pp. 368. ISBN 0-12-469521-3. \$49.50.

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Hydrazine and its Derivatives: Preparation, Properties, Applications. By ECKART W. SCHMIDT. Wiley: 1984. Pp. 1059. ISBN 0-471-89170-3. £80.75.

Index of Chemistry Films. J.S. CLARKE (ed.). Royal Society of Chemistry: 1984. Pp. 463. ISBN 0-85186-499-6. £19.00, \$34.00.

Natural Products Chemistry, Vol. 3. By K. NAKANISHI *et al.* Oxford University Press: 1984. Pp. 700. ISBN 0-19-855711-6. £60.

Natural Products Chemistry, Vol. 3. K. NAKANISHI *et al.* (eds). University Science Books, 20 Edgehill Road, Mill Valley, California 94941: 1984. Pp. 700. ISBN 0-935702-14-8. \$90.

Organic Chemicals from Biomass. DONALD L. WISE (ed.). Benjamin/Cummings: 1983. Pp. 465. ISBN 0-8053-9040-5. \$41.95.

Organic Chemistry. By G. MARC LOUDON. Addison-Wesley: 1984. Pp. 1451. ISBN 0-201-14438-7. Np.

Oxidation of Organic Compounds: Medium Effects in Radical Reactions. By N.M. EMANUEL, G.E. ZAIKOV and Z.K. MAIZUS. Pergamon: 1984. Pp. 611. ISBN 0-08-022067-3. £57.50, \$95.00.

Quantum Chemistry: The Development of Ab Initio Methods in Molecular Electronic Structure Theory. By HENRY F. SCHAEFER III. Clarendon: 1984. Pp. 144. ISBN 0-19-855183-5. £15.

Radiative Corrections in SU(2)_L × U(1). B.W. LYNN and J.F. WHEATER (eds). Wiley: 1984. Pp. 335. Hbk ISBN 9971-966-26-3; pbk ISBN 9971-966-28-X. £29.

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Water Analysis, Vol. II. Inorganic Species, Part 2. ROGER A. MINEAR and LAWRENCE H. KEITH (eds). Academic: 1984. Pp. 405. ISBN 0-12-498302-2. \$65, £45.50.

Applied Biological Sciences

Advances in Viral Oncology, Vol. 4 Mechanisms of Neoplastic Transformation at the Cellular Level. GEORGE KLEIN (ed.). Raven: 1984. Pp. 349. ISBN 0-89004-977-7. \$98.

Aplastic Anemia Stem Cell Biology and Advances in Treatment. Progress in Clinical and Biological Research, Vol. 148. NEAL S. YOUNG, ALAN S. LEVINE and R. KEITH HUMPHRIES (eds). Alan R. Liss: 1984. Pp. 357. ISBN 0-8451-0148-X. \$74.

Chemical Induction of Cancer: Structural Bases and Biological Mechanisms. By JOSEPH C. ARCOS, YINTAK WOO and MARY F. ARGUS. Academic: 1984. Pp. 780. ISBN 0-12-059303-3. \$78.

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Eukaryotic Cell Cultures: Basics and Applications. Advances in Experimental Medicine and Biology, Vol. 172. RONALD T. ACTON and J. DANIEL LYNN (eds.). Plenum: 1984. Pp. 556. ISBN 0-306-41619-0. Np.

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Research in the market-place

Dorothy Nelkin

The Interferon Crusade: Public Policy and Biomedical Dreams.

By Sandra Panem.

The Brookings Institution, 1775 Massachusetts Avenue NW, Washington DC/ Costello, 43 The High Street, Tunbridge Wells, Kent: 1984. Pp. 109. Hbk \$22.95, £31; pbk \$8.95, £12.10.

"A MAGIC bullet", "a priceless miracle drug", "a cure for cancer", "a wonder therapy" — it was in these terms that the public was exposed to research on interferon. Interferon has been the most visible and dramatic product of the rapidly expanding biotechnology industry. The story of its development and the hyperbole that surrounded it reflect the mix of scientific, political and economic events that has generally shaped the biotechnology revolution. It is a story of promises and problems, of complex arrangements between government, industry and academia, and of the policy dilemmas of a fast-moving and potentially profitable field of research.

Sandra Panem's book, *The Interferon Crusade*, begins with the discovery in the 1950s that the body produces a natural therapeutic agent, "an interfering protein" to inhibit infections. Panem traces the development of interferon, documenting the discovery of its anti-tumour activity in 1967, the publicity about its potential as a cancer cure in the mid-1970s, and finally the successful cloning of a human interferon gene in late 1979. She writes also about the role of advocacy scientists, those key individuals who publicize research, capitalizing on the public fear of cancer in order to raise funds.

As Panem observes, the explosion of interest in interferon resulted mainly from its promise as a cure for cancer. The ability to synthesize the drug through genetic engineering techniques, and therefore to produce it inexpensively, aroused commercial attention so that interferon became the primary lure for trapping venture capital for biotechnology firms. The case of interferon reveals the complex institutional relationships involved in new technologies. It engaged scientists and politicians in marshalling resources to develop the technology; it attracted the interest of the press; and eventually it succeeded in mobilizing public curiosity and venture capital. The academic community, the government and the private sector developed close relationships.

The book points to the controversial policy issues raised by these relationships — the effect of extra-scientific factors such as media attention in setting research priorities, for example, and the influence of commercial interests on academic research and on the behaviour of the

scientific community. It also deals with the conflicts over open disclosure of research findings, the manpower problems of a rapidly expanding scientific and technical field, the ethical and legal consequences of the changing relationship between science



"Forget the heroin business, Bugsy! I've something more profitable lined up!"

ACCORDING to a report prepared by the US brokerage and investment firm Bache Halsey Stuart Shields, interferon sales could have a world wide market of \$2 billion a year within three to five years. The report, summarised in *European Chemical*

Interferon fever — cartoon and news report from Nature May 1980.

and industry, and the complexity of funding patterns that arise from the involvement of both the public and the private sectors.

Panem is at her best when she discusses the history of the development of interferon, and its scientific importance in molecular biology and immunology despite its failure in the short term as a therapeutic agent. She writes skilfully of the dynamics of the biotechnology industry and the role of media, both in shaping research priorities and attracting industrial interest. The people, the firms, are real, and their activities are richly portrayed. However, she presents the key policy issues as little more than a list. There is much more to be explored in the problems of secrecy so often observed as a consequence of the new associations between science and industry engendered by the growth of biotechnology. How great a problem is

secrecy in science? What kind of contracts are being developed, and what is their effect? What is the actual influence of non-disclosure on scientific productivity?

One suspects the changing relationship between science and scientists and their sponsors is also changing the prestige system in science. Rewards are increasingly coming from consulting contracts, from participation on corporate boards. What does all this mean? At some universities entire biology departments have been redirected to research in genetic engineering and biotechnology-related research. What does this imply for the training of students, and more generally, for the wider range of biological research? There are also many questions to be asked about the selling — the overselling — of science, and Panem makes a fascinating observation about the role of "hype" on funding patterns. Between 1978 and 1981 the increase in government spending on interferon research greatly outpaced the overall rate of growth in biomedical research in general. Yet the peer review ratings were much lower, suggesting how politicians more than peers can shape scientific priorities. What are the implications of this pattern of funding "hot" research subjects before good proposals are developed? When commercial interests influence research funding, what does this do to the ideal of science as an autonomous, neutral activity? While the focus on fashionable areas of research has implications for science itself, there are perhaps more serious implications for the rest of us.

The overselling of interferon also bears on the credibility of science as a whole. In many cases, scientists contributed to media publicity, believing that it was an effective way to attract support. But this also created inflated public expectation and then disillusion as delivery of therapeutic benefits was delayed. As one journalist cynically observed about interferon, "At only a hundred trillion dollars a gram, this miracle drug has a future".

Panem competently outlines the policy dilemmas intrinsic to this revolutionary area of research, but she offers no point of view. Her descriptions of the "interferon crusade" are balanced, but bland: she takes no position on the many controversial issues that she lists. Her view, as stated in the introduction, is that "understanding the interferon experience may prove useful in predicting future problems of biomedical research [and] other scientific areas as they experience technical revolutions". She has laid the groundwork for such an understanding, but it remains for others to examine with a more critical and analytical eye the dilemmas that are outlined in her book. □

Dorothy Nelkin is a Professor in the Program on Science, Technology and Society, and in the Department of Sociology at Cornell University.

Renewed interaction

Peter D. Moore

Allelopathy, 2nd Edn.

By Elroy L. Rice.

Academic: 1984. Pp. 422. \$55, £42.50.

PERHAPS the best possible excuse for writing a second edition of a book is when the author changes his mind about the definition of the title; this becomes even more imperative when the title is a single word, such as allelopathy. When Rice wrote the first edition of this book in 1974, he adopted a conservative definition and confined his attentions to those chemical interactions between plants (including microbes) which result from the release of compounds into the environment and which cause harm to the recipient. He now feels that the definition was too narrow and that the term should include stimulatory interactions, and in this respect he comes into line not only with the coiner of the word (Molisch) but also with many others who use it.

Predictably, wider use of the term has resulted in the second edition including information which was excluded from the first. Hence it is a fatter book. But there is also a marked change in emphasis, for almost a third of this edition is devoted to what may be regarded as applied aspects of the subject, such as the importance of allelopathic studies in agriculture, horticulture and forestry. Further semantic problems occur even in these fields, as in the case of such well-known weeds as *Camelina sativa* in flax fields. It has long been known that flax plants in the immediate vicinity of the weed suffer reduction in growth rate, but is this due to allelopathy or to "competition" for some environmental resource such as nutrient ions? Both views have been held by various workers and chemical tests on plant exudates have not clarified matters, for leaf washings of *Camelina* have often been found to stimulate the growth of young flax plants. The latest work quoted by Rice suggests that benzylamine, secreted by *Camelina*, stimulates flax at concentrations below 100 ppm, while above 200 ppm it serves to inhibit it. So here we encounter problems both in the definition of allelopathy and of competition which are currently dogging the study of this subject.

In the analysis of allelopathic interactions in natural vegetation, emphasis has often been placed upon the occurrence of bare patches around plants or of patterns exhibited by plant populations. This work is thoroughly reviewed here, both for herbaceous and for woody species. The involvement of microbial elements in the

plant interactions also receives due attention, as for example in the discussion of nitrification and the extent to which late successional species produce chemicals which inhibit the process and thereby gain advantage over those species with the potential to replace them. This is a subject which generates strong feelings among ecologists and it is well reviewed by Rice, who, predictably, comes down in favour of the idea of succession suppression by dominant plants.

From the ecological, we are taken into the realms of physiological and biochemical studies of allelopathy, including the chemical nature and the mode of transfer and action of the active agents.

The subject of allelopathy is one which transgresses a number of disciplinary boundaries and which can be expected to interest a wide readership. The first edition of this book certainly spent more time off my shelves than on them and is now very well worn. Such intensity of use, which I am sure is widespread, coupled with the amount of research into allelopathy over the past ten years, amply justifies the publication of a second edition even if the author had not revised his opinion on the definition of the term. □

Peter D. Moore is Reader in Ecology in the Department of Plant Sciences, King's College, University of London.

Ice age development

G. de Q. Robin

Ice Sheets and Climate.

By J. Oerlemans and C.J. van der Veen.

Reidel: 1984. Pp. 217. Dfl. 90, \$34.50.

Ice Sheet and Climate Modelling would have been a better title for this work, which aims to help graduate students bridge a gap between glaciology and climatology. The authors' views and research contributions are presented concisely in relation to brief outlines of our knowledge of the climate system and of the work of other modellers.

In 12 short chapters descriptions and relevant equations are presented, starting with the global climate system. Here the energy balance, transport by atmosphere and oceans, and related effects of albedo feedback on these systems are covered. A largely mathematical outline is given of ice dynamics, including flow of ice shelves and grounding lines problems. Economical methods of numerical modelling are then introduced, and are later applied to problems of growth, decay and stability of ice sheets in the Northern Hemisphere and the climatic stability of ice sheets of Greenland and Antarctica.

Adequate modelling of vast ice sheets must involve their heat budget and bedrock adjustment to ice loading. Oerlemans and van der Veen avoid lengthy analysis of frictional heat production from stress-strain rate distribution by equating the loss of potential energy to total frictional heat production in steady-state ice sheets. Their analysis of bedrock adjustment is similar to that found earlier works, but leads to more sweeping conclusions.

In the book, the approximate 100,000-year cycle of growth and decay of Pleistocene ice sheets is attributed to flow dynamics of the asthenosphere and ice sheet once growth is initiated. The authors place less emphasis than others on latitudinal variations of radiation with time as a controlling factor. Even so they consider such variations are one likely cause of initiation of ice ages, with the next suitable minimum at 65°N at 50,000 years being

much more likely to start an ice age than the earlier but less severe minimum around 20,000 years. In the near future, they calculate, warming of the atmosphere by doubling of the carbon dioxide content will deposit more ice on Antarctica (where melting is negligible) while on Greenland total melting would increase more than accumulation. The assumption that ice flow changes are negligible over a short period leads to estimated mass changes of the two ice sheets that together would lower sea level by 0.1m in 250 years.

When linking heat flow to large-scale ice dynamics, Oerlemans and van der Veen reach similar conclusions to other studies but with two exceptions. They reject internal strain heating of cold ice and frictional enhancement of sliding by ice melting as likely causes of major ice surges, pointing out that some conditions that lead to surging of valley glaciers do not apply to ice sheets. They do however conclude that basal trapping of water beneath ice sheets could result in large-scale cyclic surging. At this point, in moving on to develop models that could work rather than first discussing if these are plausible, it seems that the cart is put before the horse. Surely beneath fast-moving ice streams, sometimes termed "continuously surging glaciers", water trapping must be stable and continuous rather than intermittent. Since such ice streams discharge a large fraction of Antarctic ice, the authors could query whether really large-scale surges are possible. Neither modelling studies nor field data seem to provide strong evidence in their favour.

Although it is apparent that English is not the authors' mother tongue, the book is clearly written and presented. Its broad conclusion is that ice-asthenosphere dynamics drive an ice age — once it is triggered off — rather than climatic factors driving ice sheet dynamics. Oerlemans and van der Veen do not claim to provide final answers, but they have produced a stimulating book that should, as they intend, result in further and more detailed research in this field. □

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● Cambridge University Press has issued paperback editions of *Theory and Experiment in Gravitational Physics* (by Clifford M. Will) and *The Very Early Universe* (edited by G.W. Gibbons, S.W. Hawking and S.T.C. Siklos). Price of each book is £15, \$24.95. For reviews see *Nature* 299, 284 (1982) and 309, 473 (1984).

Remains of humanity

Yoel Rak

The Origins of Modern Humans: A World Survey of the Fossil Evidence.
Edited by Fred H. Smith and Frank Spencer.

Alan R. Liss: 1984. Pp.590. \$70, £53.

IN THE late 1930s only the fact that a group of human fossil remains was discovered at the same site on Mount Carmel spared each specimen from being assigned to a different taxon — a “separate form of humanity”. There can be no better manifestation of the changes in approach and thought regarding the appearance of anatomically modern *Homo sapiens* than the articles offered in *The Origins of Modern Humans*. There has long been a need for such a volume, to bring together data on the enlarged fossil record and demonstrate the innovations in our methods of assessment of that record. One is reminded that almost 30 years have passed since the appearance of the last attempt to summarize the state of the art in this area, *Hundert Jahre Neanderthaler 1856–1956*, published in commemoration of the hundredth anniversary of the first Neanderthal discovery.

Of the 11 contributions in the book, the first two are of a general nature. F. Clark Howell introduces matters with a comprehensive survey that in essence summarizes the contents of the whole volume. And as historical background and past ideologies must be considered in any attempt to assess theories of the appearance of modern humans, the second chapter, written by F. Spencer, concentrates on the history of the fossil discoveries and their interpretations. It is unfortunate, however, that the three main evolutionary models of early *H. sapiens* so clearly depicted by Spencer are again independently described several times in following chapters, creating a rather tedious redundancy.

The heart of the book consists of the nine remaining contributions, which are devoted to the skeletal remains found in various regions of the Old World and the New: Europe (covered by C.B. Stringer, J.J. Hublin, B. Vandermeersch; F. Smith; and D. Frayer); western Asia (E. Trinkaus); Africa (P. Rightmire, G. Brauer); east Asia and Australasia (M.H. Wolpoff, Wu X.Z. and A.G. Thorne); China (C.L. Brace, Shao X-q. and Zhang Z-b); and the Americas (R.C. Owen). The articles are to a large extent organized according to a similar pattern. Initially there is an up-to-date account of the fossil record and its chronology, thus allowing the reader to become acquainted with a number of little-known specimens, that being followed by interpretation of the fossils, primarily phylogenetic. The geographical scope of the book as a whole is both broad and well-balanced; as such it is refreshingly free of the Eurocentric bias that, as the editors

remind us, formerly plagued anthropological thought. Similarly, the range of approaches and opinions of the various authors reflects most of the current thinking on the topic. Only by close attention to the differences in approach to morphology and to the differing biological models of each author will the reader comprehend how the same group of specimens is considered “archaic”, “primitive” and “generalized” by some, and “advanced”, “specialized”, “derived” and “modern” by others.

The views of most of the contributors are already well known, but publication of their current research in one volume provides for the first time an overall perspective on the appearance of anatomically modern *H. sapiens*. Because of the range and diversity of the contributions, the references accumulated here by themselves constitute a veritable treasure trove.

Still, there are some criticisms. Detection of the polarity of a morphocline in the period under discussion is admittedly difficult because of the relatively short time span involved, the polymorphic nature of the species, and, lastly, the fully modern volume of the brain in the hominids considered (a criterion which had dictated polarity in earlier stages of human evolution). Nevertheless, more of the contributors could have attempted to deal with the question of evolutionary trends through a more rigorous functional interpretation of particular morphologies. Culture with its effects on morphology is the primary topic of those who do offer an adaptive interpretation. Such an approach, along with precise dating, might ensure that a greater degree of certainty could have been accorded the somewhat speculative statements on migration, local development and replacement.

The importance and overall quality of the book are not seriously marred by several other deficiencies. A world map showing all the sites mentioned would have been most useful (the two maps which do indicate sites unfortunately portray the same region and most of the same fossil locations), while the quality of some of the photographs and drawings leaves much to be desired. A greater effort could have been made to standardize the names of fossils and sites; the skull from Saldanha, for example, appears under three different names. Finally, although the book deals with the fossil evidence, some reference to genetic studies would have been in order.

The editors and contributors to this book have provided us with an enlightening review of the problems surrounding the origins of anatomically modern *H. sapiens*. Readers will need some prior familiarity with the fossil record, and with anatomy and population genetics. For such people this will be an invaluable source of reference. □

Yoel Rak is a Lecturer in the Department of Anatomy and Anthropology at Tel Aviv University, Israel.

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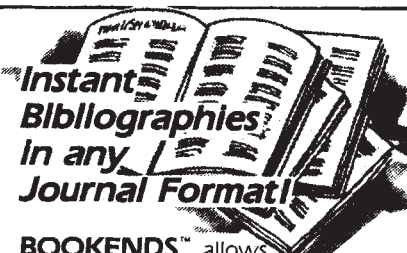
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Reader Service No.15

Ultrastructuralism

Michael Ashburner

Insect Ultrastructure. Vol. 1 The Ultrastructure of Gametes. Vol. 2 The Ultrastructure of Developing Cells.

Edited by Robert C. King and Hiromu Akai.

Plenum: 1982/1984. Vol. 1 pp.624, \$55, £52.25. Vol. 2 pp.650, \$85, £80.75. Two-volume set \$127.50, £121.13.

In September 1969 the Royal Entomological Society of London held a symposium called "Insect Ultrastructure". Of the eleven people who contributed to it five were, or had been, members of the Department of Zoology in Cambridge. Surprising, considering that the meeting was organized by an Oxford man. The present two volumes have 49 contributors, only one of them from Cambridge Zoology. But then only one, David Spencer Smith (to distinguish him from all the other eminent biologists, let alone Oxford professors, called David Smith), has contributed to both of these volumes and to the 1969 symposium. David Spencer Smith is, now, in Oxford.

Who will read such volumes as these, 31 chapters on the ultrastructure of insects? I suppose there are those who will read anything on structure if it is ultra, whether from bees or bats. There are, I know, those who will read (almost) anything on insects, from ultrastructure to sociobiology. For these readers the two books suffer from a serious lack of balance. Some of the chapters are very narrow, others paint on a broader canvas. For example at one extreme we have Sorsa's review of the ultrastructure of the polytene chromosomes of *Drosophila melanogaster*. I have no quarrel with Sorsa's chapter as a contribution to science (at least no quarrel worth airing in these pages) but the ultrastructuralist or entomologist reading these volumes should be warned that it is very much a review of Sorsa's own work and makes no attempt to review the field comprehensively. Moreover there can be few who require 10 pages of a detailed map of the banding pattern of chromosome arm 2L. An \$85 book is not a suitable medium for its publication.

Another example of a review whose field is narrow is that by Rasmussen and Holm on the ultrastructure of meiotic prophase in the silkworm, *Bombyx mori*. I am sure that this is a subject worthy of review. That is not my complaint. My complaint is that in a large and expensive work called *Insect Ultrastructure* the purchaser might expect a more comprehensive review of, for example, meiotic prophase in insects or, perhaps, of meiosis in *B. mori*. I must not

give the impression that all of the chapters are as narrow in their compass as these. Many, indeed, will be of interest to biologists with a wide range of interests, David Spencer Smith's review of the ultrastructure of insect muscle being one such.

In addition to the two classes of omnivorous reader there are those (and here I include myself) who approach volumes like these with the attitude of a selective browser. We will read chapters in this book for one (or more) of four reasons: (i) because we need to know what is going on in a particular field of research (which may be our own); (ii) because we always wanted to know about, for example, neuromuscular junctions in the Orthoptera but never, before, knew who to ask; (iii) because we are intrigued by the title of a particular chapter; or (iv) because we once met Professor Kafatos at a conference banquet in East Idaho and always wondered what he did for a living. For the selective browser balance is irrelevant; these volumes are a lucky dip to which he can return over and over again.

It is interesting, for an outsider whose practical experience of insect ultrastructure is limited to peering over Nancy Lane's

shoulder at a glowing screen, to compare Neville's 1969 volume and David (Spencer) Smith's 1968 book *Insect Cells: Their Structure and Function* with these volumes. Two overall impressions. The first is the importance of technical advances: not only that the slow but steady improvement in preparative techniques has led to far better electron micrographs, but of methods such as scanning electron microscopy (in its infancy in 1968), freeze fracture, chromosome spreading and reconstruction from serial electron micrographs that are now routine tools. The greatest contrast, though, is that the two earlier books, Smith's especially, left one with a very static view of structure, making the necessary relationship between structure and function difficult to draw. Now, however, I get a real feeling for dynamic processes, of the importance of the change in structure, and hence function, with time. It is impressive to see how much the study of insect ultrastructure is now the adjunct of the experimental biologist, rather than the preserve of the morphologist. □

Michael Ashburner is Reader in the Department of Genetics, University of Cambridge.

Sea slugs: beauty in biology

Elizabeth B. Andrews

Biology of Opisthobranch Molluscs, Vol. II.

By T.E. Thompson and G.H. Brown. *The Ray Society, British Museum (Natural History), London: 1984. Pp.229. £39.*

THE delicacy of shape and the beauty of colouring of sea slugs, the animals with which this volume deals, have captured the attention and admiration of many zoologists, amateur and professional. Their agility and grace of movement contrast markedly with the drabness and ponderous crawling of their terrestrial relatives and with their own dreadful deadness when pickled. They have remained a rather neglected group by comparison with both prosobranch and pulmonate gastropods, however, due partly to their unreliable occurrence and partly to the fact that many species are particularly abundant in those marine habitats that are most difficult to explore — too low for shore collecting, too shallow for adequate boat-work. The first difficulty still remains, and some species are genuinely rare. Scuba diving has largely abolished the second, however, and one reason for the success of this book is that both authors are accomplished divers and have thus found not only more specimens for study but also more species new to the area covered by their work.

The authors offer a taxonomic survey of

the nudibranch gastropods found in British waters, with keys and illustrations to help identification. They give brief definitions of subordinal and familial characters, and for each species provide details of appearance, anatomy, feeding, breeding and a brief synonymy. Local distribution is recorded in a series of maps, and foreign distribution is mentioned, and there are short discussions of taxonomic problems, a very complete list of references and a helpful index. The book also benefits from the provision of an "Epilogue": a summary of ideas and information relating to opisthobranchs published since 1976 when Vol. I appeared.

Illustrations are important in any book dealing with such photogenic subjects as nudibranchs, and comparison with *The Ray Society's* previous publication on the group, Alder and Hancock's *A Monograph of the British Nudibranchiate Mollusca* (1845–1855), with its beautiful figures, is inevitable. Thompson and Brown's work stands up to the comparison with distinction: all but two species are illustrated in a series of plates on which paintings of the animals stand against a black background, lifelike and attractive. In addition, each species is shown in black and white, a helpful guide for the student.

For all interested in this group of animals the book will be an essential acquisition. It is an excellent addition to the series which *The Ray Society* has produced for so long, and a worthy successor to Alder and Hancock's *Monograph*. □

Elizabeth B. Andrews is Senior Lecturer in Zoology at Bedford College, University of London.

● Next week's issue of *Nature* includes the Spring Books Supplement, 25 pages of reviews from, among others, W.F. Bynum, Richard C. Lewontin, Nevill Mott, David Park, Owen Gingerich, David Bohm and Anthony Clare.

New for FASEB at Anaheim

This year's meeting of the Federation of American Societies for Experimental Biology is at Anaheim, California, opening on 21 April.

● Visitors to Vector Laboratories at Booth 2027 at FASEB can obtain the newest reference and applications list for the Vectastain ABC technique. Citing some 120 of the most recent publications using this ultrasensitive biotin/avidin-based immunoperoxidase system, this list is the third in a series. In addition to providing a compilation of current references, an index of specific applications is included, highlighting pertinent articles using lectins or monoclonal antibodies for immunohistochemistry, enzyme immunoassays, nitrocellulose blotting, DNA hybridizations and electron microscopy.

Reader Service No. 100.

● Two high performance gel filtration columns, Superose 6 HR 10/30 and Superose 12 HR 10/30, have been introduced by Pharmacia. Superose is a unique, cross-linked agarose matrix with high chemical stability over a pH range of 1–14. Prepacked in high resolution glass columns (1 × 30 cm), Superose provides high protein and enzymatic recoveries, reproducible separations and long column life. The two products cover a molecular weight range for globular proteins of molecular weights between 1,000 and 5 million.

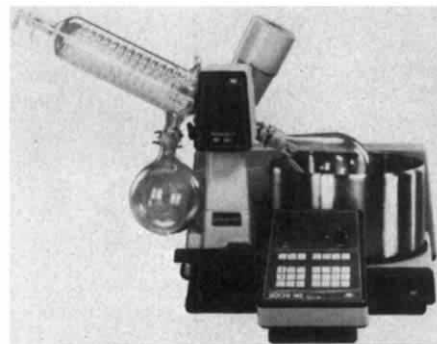
Reader Service No. 101.

● At the 1985 FASEB show, Bio-Rad Laboratories (Booth 2220) will feature the new Protean II vertical electrophoresis system, a new chromatography system for preparative scale monoclonal antibody purification, and new protein micro-analyser. In addition, a variety of chromatography and electrophoresis products of special interest to life scientists are on view. These include: HPHT-MAPS monoclonal antibody purification system for removal of protease, transferrin, albumin, and other contaminating proteins from up to 20 mg of mouse, human, and rat IgG and IgM. The Quick-Check analyser for purification monitoring or protein profiling in 15 min. A protein chromatography system with six complementary chromatographic mechanisms to produce highly purified, active proteins. A data acquisition system with software reintegration, peak naming, and data storage. A fixed-wavelength UV monitor for high sensitivity detection at 280, 254, and 214 nm. A new video densitometer allowing data to be viewed, manipulated, and optimized directly on the video screen. And Immun-Blot, a range of non-isotopic picogram-sensitive blot detection kits.

Reader Service No. 102.

● A new ion-exchange medium that can be packed more quickly and easily than conventional gels, and allows faster flow rates for high-speed protein isolations, is being introduced by Waters Chromatography Division of Millipore Corporation at the FASEB/ASBC meeting. On view on Booths 2633–2641 and 2732–2740, Waters Accell ion-exchange medium is a silica-based chromatography packing featuring a patented copolymerization process that exploits the properties of a rigid separation medium while giving excellent recovery of biological activity. The patented copolymerization process encapsulates the rigid wide-pore silica base to produce low, non-specific binding. The rigid structure permits linear scale-up at all flow rates and pressures, from analytical to preparative to process chromatography, using the same methods and chemistry.

Reader Service No. 103.



Two from the Brinkmann booth, the Buchi rotary evaporator (top) and Brinkmann's digital diluter.

● At Booths 2500–2505, Brinkmann Instruments Co. is showing the new Buchi medium pressure liquid chromatograph for preparative-scale isolations and Brinkmann homogenizers for use in the industrial or biomedical laboratory. Brinkmann is also introducing the new Buchi rotary evaporator Model RE-140, which provides full automatic evaporation via a microprocessor-controlled keyboard. Other products include the Brinkmann digital diluter 9200, featuring continuous selection of volumes from 1 μ l to 10 ml and fractional volumes to 0.1 μ l.

Reader Service No. 104.

● Pierce Chemical's Diphenyl-8 GC capillary column conditioner is specially purified diphenyltertramethyl-disilazane (DPTMDS). With the higher temperatures and increased sensitivities common to capillary column work, the presence of silanol groups on the stationary phase and column can cause major chromatographic problems. Diphenyl-8 eliminates the symptoms characteristic of residual silanol, prolonging the lifetime of a column by reducing stationary bleed. It deactivates silanol groups on the solid support and acts as a "cleaning agent" for column residues capable of being derivatized.

Reader Service No. 105.



The new Orion cyanide standard.

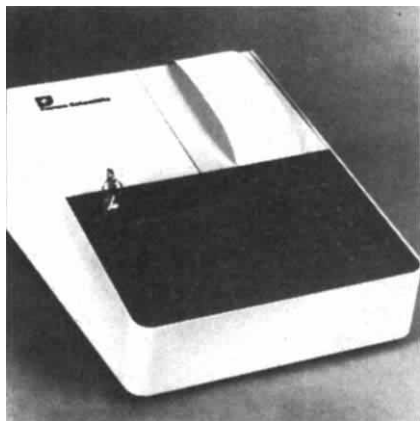
● Orion Research Incorporated has introduced convenient packs of cyanide standard for use with cyanide electrodes as a calibrating solution. Each reagent pack contains 10 sealed glass ampules, with 50 ml of 100 p.p.m. cyanide standard in each ampule. The standard can be used directly as a 100 p.p.m. calibrating solution or diluted to provide additional dilutions.

Reader Service No. 106.

● The J.T. Baker Chemicals range for lipid screening has been expanded by the addition of two new reagents for cholesterol and triglycerides monitoring. These reagents are formulated according to the CHOD-PAP and GPO-PAP principles respectively. The cholesterol reagent is in a liquid, ready-to-use configuration. The reagent remains stable for up to 12 months and shows linearity up to 20 mmol l⁻¹. The triglycerides reagent is lyophilized and is stable on reconstitution for 3 weeks at 2–8°C. Application sheets for lipid screening reagents are available for a variety of clinical chemistry analysers.

Reader Service No. 107.

These notes are based on information provided by the manufacturers. Use the reader service card bound inside the journal to obtain further information.



● Forma Scientific's new desk-top programmable digital recorder/alarm monitor, the Model 1525, can receive input from, and record any combination of, eight conditioned millivolt signals, such as temperature, per cent CO₂ and O₂, per cent RH, and pH. The sensitouch keyboard makes it easy for the user to program the high/low deviation limits for any specified condition. The ink printer records date, time, function, and input number, plus a printout at programmed intervals. Additional printouts are furnished along with audible and visual alarms in the event of a deviation beyond setpoint limits.

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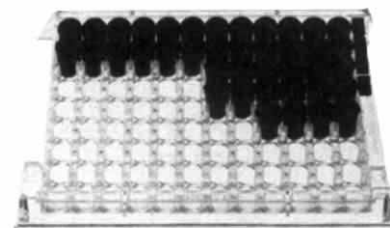
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Removawell, Dynatech's new plate holder.

● Dynatech Laboratories has added a new Removawell plate holder for Microtiter and Microelisa plates. The plastic disposable Removawell system allows the user to build up an 8 × 12 well plate from strips of wells or from individual wells. Up to 96 wells in total can be held firmly in any configuration. The Removawell holder is designed so that it may be inverted with complete safety when emptying the contents of the wells, regardless of the number of strips or wells present. When assembled with wells, the whole Removawell system has the rigidity and strength of a conventional Microtiter plate, and it may be stacked for easy storage.

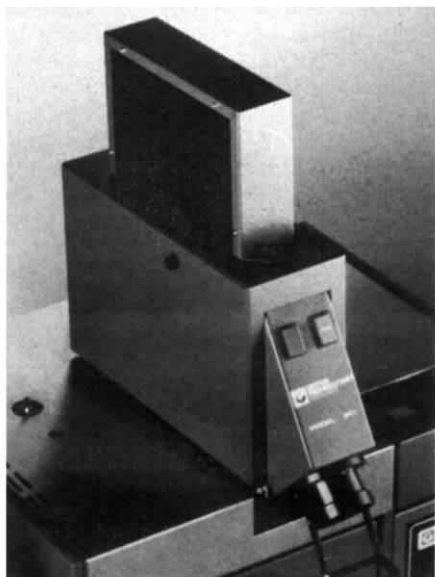
Reader Service No. 109.



A splash-proof touch-sensitive keypad controls the WPI pH meters.

● The 700 Series microelectrode amplifiers from World Precision Instruments are designed principally for intracellular recording using fluid filled, high impedance glass microelectrodes. All units feature ultra-stable, gold-plated input probes, low noise, negative capacitance and electrode test capabilities. Applications include intracellular and extracellular potential measurements, impedance and ion selective measurements. Also new from WPI, the HI 8417 and HI8418 microprocessor-based pH meters are programmed for easy daily laboratory use. Both meters feature automatic recognition of three different buffers and automatic temperature compensation. Two large LCD displays allow for simultaneous viewing of pH (or mV) and temperature. The HI 8418 includes a printer and a numeric keypad. The printer can list not only the pH/mV readings, but also the time, date and calibration parameters. The numeric keypad can be used to enter user-defined buffers and intervals for timed printing of data.

Reader Service No. 110.



Robotics makes Packard's new sample injector simple to use, with both packed and capillary chromatography columns.

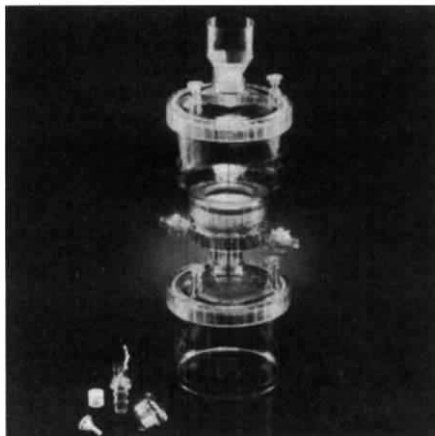
● Packard's "One-Shot Samplers" are injectors for gas chromatography that offer operational simplicity and security. The operator needs only to position a sample vial and press the "start" button to effect injection and begin the analysis. Because the sample volume injected is accurately and reproducibly measured, the use of the external standard (ESTD) method becomes much more attractive; in many cases it can replace the internal standard method (ISTD). Two models are available, the Model 941 can inject into either the front or rear injection port. The desired port is software selectable, this feature facilitates the automation of identification and duplication of analyses. Two different analyses can therefore be run. The Model 940 is similar but allows injection only into the injection port above which it is mounted. Both models are suitable for packed and capillary columns.

Reader Service No. 111.

● New England Biolabs have developed an *in vitro* method for creating novel DNA cleavage specificities. This simple procedure involves the methylation of the DNA with a site-specific methylase followed by cleavage of the DNA with a restriction endonuclease whose recognition sequence overlaps that of the methylase. Only a specific subset of the restriction endonuclease sites will be cleaved (see *Nucleic Acids Res.* 5, 5165-5173; *Biotechniques* 2, 218-222). The latest additions to New England Biolabs' list of over 90 restriction endonucleases include the following: *BsmI* (GAATGCN/); *FspI* (TGC/GCA) (= *MstI*); *NheI* (G/CTAGC); *SspI* (AAT/ATT); *SpeI* (A/CTAGT); and *StyI* (C/C(A,T) (A,T)GG). New DNA methylases include; *M.HphI* (TCACC); *M.BamHI* (GGATCC); *M.HaeIII* (GGCC); and 'dam' (GATC). New M13 cloning vectors from NEB are: M13mp18 RF I DNA and M13mp19 RF I DNA. Reader Service No. 112.

● The new polymer-based columns from Hamilton Co. of Reno, New York, are intended to extend the limits of HPLC and ion chromatography. Hamilton polymeric reversed-phase (PRP) resins accept eluents between pH 1 and 13, whereas silica tolerates only pH 2 to 8. For HPLC, PRP-1 columns separate virtually all classes of compounds, with no need for ion-pairing reagents. Because PRP particles are chemically identical from surface to centre, selectivity variations common to silica cannot occur. Columns are available with different particle sizes (5 and 10 micrometres) and in a variety of lengths (15, 25 and 30.5 cm) to meet most chromatographic needs. Low-cost bulk packing is available for preparative-scale separations. For ion chromatography, the PRP-X100 achieves rapid separation of the common anions. Unlike silica, the pH tolerance of PRP-based resins permits high-resolution separations of cyanide, sulphide, silicate, borate, and other anions requiring high-pH mobile phases. The 10-micrometre PRP-X100 columns are compatible with existing HPLC and ion chromatography equipment, and are available in 10, 15, and 25 cm lengths.

Reader Service No. 113.



On the Vanguard booth, the latest Sartorius filter holder.

● A multipurpose filter holder, manufactured by Sartorius GmbH, is now available in the United States from Vanguard International. Applicable for vacuum or pressure filtration, the multipurpose filter holder is integrally moulded of clear polycarbonate for strength and to ensure repeated autoclavability at 121°C steam. Applications for the filter holder include clarification and/or sterilization of liquids, and microbial analysis such as sterility testing.

Reader Service No. 114.

● A new brochure available from Tekmar describes the company's line of high shear dispensers/homogenizers. Units are available in sizes from laboratory to full-size production. Standard machines are available for batch and in-line processing of liquid/liquid, solid/liquid and gas/liquid fluids. Special metal alloys and standard unit designs are also available.

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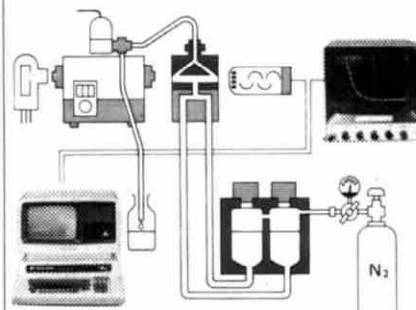
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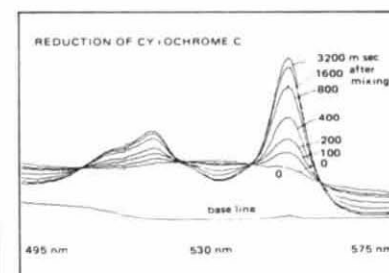
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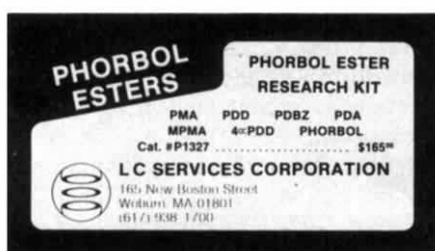
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● The K531 Dako PAPA kit System 20 is designed as a research tool for the demonstration of myelin basic protein (MBP) using the peroxidase anti-peroxidase staining technique. The primary antibody in this kit represents a pool of optimal antisera selected from many immunized rabbits. This polyclonal antibody shows very good reactivity with the antigen even under the adverse conditions encountered after tissue is fixed in formalin. In addition to 7 ml of primary antibody, a comprehensive set of reagents is provided in working dilutions. Five control slides for the initial use of this kit are also included. K531 has a minimum capability of twenty tests. This routine system comes with simple instructions for those with limited or no previous experience with the immunoperoxidase technique.

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Cherry, pine and clove scents overcome less attractive odours—Odo-Clave from Bel-Art.

● The unpleasant odours emitted during autoclaving can be countered using Bel-Art Products' Odo-Clave deodorant disks. Two new scents have been introduced, pine and cherry. Each 1½-inch disk is impregnated with a special heat released deodorant specifically designed to be activated when exposed to the high temperatures of the autoclave. The pine and cherry scents, like the original clove scent, all serve to neutralize, not mask unpleasant odours.

Reader Service No. 117.



The Cavo sample processor and diluter gain flexibility with a new hand-held probe.

● Cavo Scientific Instruments, Inc. has introduced a disposable-tip hand probe for use with their automated IQ 190 sample processor and Model 1500 pipettor/diluter. The hand probe ensures zero cross-contamination and is ideally suited for applications using enzymes, radioisotopes or bio-hazardous materials. The hand probe can be used for liquid transfer, or can pipette and dilute in a single operation. Multiple dilutions (serial dilutions) can also be performed using a single tip. Aspirations up to 75-microlitres can be performed using 200-microlitre disposable tips. Dilution ratios are easily controlled by adjusting the dispense volumes of the two syringe pumps on the instrument. The natural grip, weight and angle of the hand probe are optimized for ease of use and to minimize fatigue. Because the hand probe is used with an automated pipettor/diluter, variable results normally associated with extended use and operator-to-operator variations are eliminated.

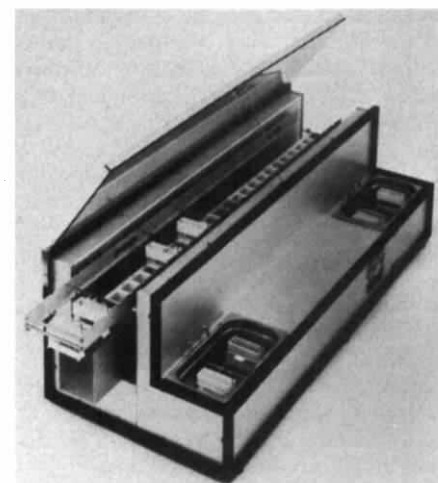
Reader Service No. 118.

● A new applications note from Amicon, entitled "DNA Concentration and Desalting Data" covers the processing of dilute DNA solutions by means of simple centrifugal ultrafiltration with Amicon's disposable Centricon microconcentrators. Concentration and desalting of plasmid DNA and plasmid restriction fragments is reviewed. Ask for Publication No. 532.

Reader Service No. 119.

● Streptavidin and biotin are being used increasingly as bridging reagents for detection of proteins, cells and nucleic acids, as well as isolation of cells and proteins. Streptavidin exhibits the same high biotin-binding capability as does egg white avidin, but with much less nonspecific binding. The result is high specificity and sensitivity with very low levels of background. Bethesda Research Laboratories has compiled a comprehensive illustrated guide, "Streptavidin: Applications in Detection of Proteins, Cells and DNA". This free 12-page booklet begins with an explanation of the powerful and versatile streptavidin/biotin interaction. The guide details many applications of streptavidin/biotin methodology, including: detection of proteins by immunoassay and immunoblotting techniques; detection of cells by light, fluorescence and electron microscopy; nonradioactive detection of DNA at 2-5 picogram levels; isolation of cells by fluorescence activated cell sorter; and isolation of proteins by immobilized streptavidin.

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The Hacker slide-stainer.

● For histology and cytology, the Hacker Instruments continuous slide stainer features linear operation through 18 stations. Immersion time is variable between 6 and 12 min, with a drain time of 3-60 s. The holders transport 22 slides each through the stations, and up to 5 holders store before and after processing. Holders may be agitated and stations 2-17 are plumbed for running water. Throughput is up to 600 slides per hour at a dwell time of 2 min per bath. All conventional and special stains and rinses can be used to give uniform standardized slides.

Reader Service No. 121.



Electroblotting is one application of the Hoefer PS 250 power supply.

● The PS 250, Hoefer's new 250-volt power supply, delivers the high current needed for fast, effective electro-transfers. It supplies currents as high as 2.5 amps. It also delivers ample voltage and current for running multiple vertical or submarine slab gels of miniature or standard size. The PS 250 has six sets of output jacks wired in parallel so that current may be drawn by as many as six units. It can operate in either constant current or constant voltage modes, with automatic crossover between modes. Full voltage and current are available for a total output of 625 watts. The PS 250 is particularly useful for electroblotting and rapid screening.

Reader Service No. 122.

● The Biomedical Technologies, Inc. range of immunoassay kits now includes RIA kits for both cyclic AMP and cyclic GMP. Each kit utilizes highly specific primary antibodies preconjugated with a second antibody to minimize nonspecific binding and to eliminate a second incubation step. The assay sensitivity for the cyclic AMP kit is 0.1 picomoles per tube and 0.005 picomoles per tube for acetylated samples. For cyclic GMP, sensitivity is 0.2 picomoles per tube and 0.005 picomoles per tube for acetylated samples. The protocol includes only three pipetting steps and one incubation and generates a visible precipitate. All necessary reagents including a true nonspecific binding reagent and acetylation reagents are included in each 200-tube kit. The kits have a shelf life of ten weeks at 4°C.

Reader Service No. 123.

● The new HP7673A automatic injector from Hewlett-Packard meets the needs of laboratories that require high chromatographic precision but do not necessarily need large volume sample throughput. The injector handles an automatic sequence of up to three liquid samples, with injection parameters regulated by a built-in controller, or through the HP3392A integrator or the HP5880A gas chromatograph. It may also be configured into a fully automated laboratory system, linking into the HP instrument network (INET).

Reader Service No. 124.



The latest Cahn instruments interface to IBM's small computer.

● The power of the IBM PC can now be used to analyse data gathered with the Thermogravimetric System 113 and Recording Electrobalances made by Cahn Instruments, Inc. The Cahn IBM Data Interface plugs quickly into the IBM PC allowing users to select data input parameters and output options. Weight and temperature data are converted to digital signals via the interface, which works with a flexible, user-friendly software package. Menu-driven commands provide choices for data gathering and output via printer or plotter. During the sample run, actual weight and temperature data are displayed in real time on the IBM monitor. A two-colour plotting routine that operates with the Hewlett Packard 7470A plotter offers multi-plot capability with graphics expansion and contraction features. Software is written in IBM basic.

Reader Service No. 125.



Up to four solvents can be blended by Perkin-Elmer's delivery system.

● Delivery systems in which up to four solvents may be blended can be used in the analysis of a wider range of samples than two or three solvent systems. This versatility is characteristic of Perkin-Elmer's four-solvent delivery system for high performance liquid chromatography, the new Series 400 and the proven Series 4. The Series 400 can be used for all types of HPLC — microbore, analytical, high speed and semipreparative. Dedicated single-function command keys simplify operation of the Series 400, including its multi-step multi-method programming. A method consists of up to ten programming steps. Nine user-defined methods can be stored and all stored data is protected by battery backup. Low conversion volumes allow solvent compositions to be changed in seconds. The Series 400 pump can be programmed for isocratic or gradient analysis with up to four solvents. Automatic compressibility compensation and positive volume proportioning, which automatically corrects for solvent mixing effects, ensure excellent reproducibility.

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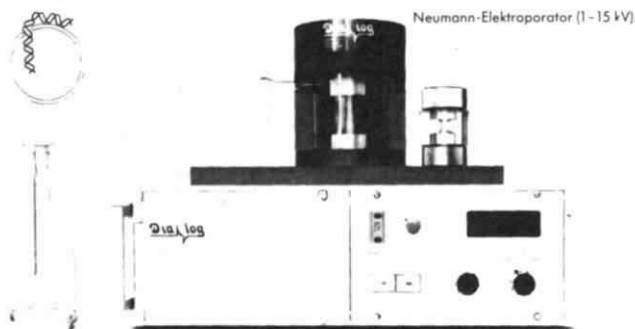
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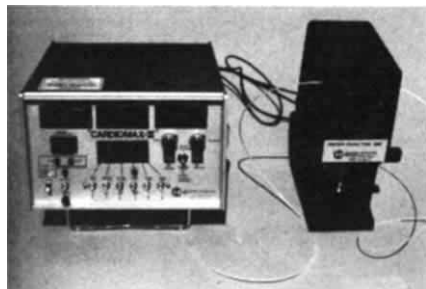
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Reader Service No. 164



Cardiac output determination — the Columbus 400.

● The New Columbus Instruments Model 400 thermomodulation micro-injector, on Booth 1429 at FASEB, has the capacity to inject rapidly 100 microlitres to 10ml of cold indicator for cardiac output determination. It is driven by electric motor and therefore does not require compressed air or CO₂ cartridges. It automatically refills itself with fresh supply of injectate through the two-way valve and volume of injectate is adjustable in steps by change of glass syringe and continuously via a precise adjustable stop.

Reader Service No. 127.

● FMC Bioproducts' SeaKem genetic technology grade agarose is prepared for use with very high molecular weight DNA, 1kb to native. SeaKem GTG agarose meets rigorous specifications for low inhibition in genetic studies and biotechnology research. High gel strength means that low concentrations can be used (0.1–1.5%).

Reader Service No. 128.

● A new line of economy chromatography columns from Kontes uses uer lock end fittings and threaded reservoir caps, eliminating typical slip-fit connections. Flex-columns are suitable for gel filtration, ion-exchange, affinity and adsorption chromatography in aqueous systems. Flex-columns are constructed by shrinking molded polypropylene end fittings onto precision ground, borosilicate column ends. A 20-micrometre bed support is compatible with a broad range of column packing bead sizes. An extensive array of column diameters (0.7cm–2.5cm) and lengths (4cm–170cm) is available, and flow adapters are available for 1.0, 1.5 and 2.5cm diameter columns.

Reader Service No. 129.

● Opti-MEM I, now available in North America from GIBCO Laboratories, is a high-performance multi-use reduced serum medium. The new medium supports the fusion, cloning and growth of human and mouse myelomas and established hybridomas. In addition, cells routinely cultured in serum-supplemented medium may be transferred directly into Opti-MEM I with a minimum of 50% reduction in serum. Opti-MEM I supports growth of: diploid fibroblast cell lines, primary fibroblasts, rat and hamster embryo cell lines, monkey kidney cells and human and bovine embryonic kidney cells. Opti-MEM I was developed by Hybridoma Sciences, Inc., Atlanta, Georgia.

Reader Service No. 130.

● The Wheaton Omnisense/Omni-Pette is a highly versatile automatic dispensing system featuring the Omni-Pette remote control dispensing head. The Omnisense comes complete with a variable speed peristaltic pump with a reverse flow option for dispensing or aspirating operations. Two models with two dispensing ranges are available: 0.25 ml to 20 ml; 0.5 ml to 840 ml. Both models feature digital volume and speed controls and also a five-digit dose counter with reset and audible cycle alert. Combined with the Wheaton Omnisense, the Wheaton Omni-Pette remote control dispensing head allows the operator to activate the dispensing cycle at some distance from the Omnisense by means of a micro-switch located on the handle of the Omni-Pette.

Reader Service No. 131.

● A new 14-page brochure describes the full line of Zeiss Invertoscopes for transmitted-light applications. The brochure includes the Axiomat IDC, the top-of-the-line Zeiss research microscope that integrates two fully automatic 4 × 5 inch and 35mm camera systems with internally corrected optics and mechanical and thermal stability. The Axiomat features a flexible, modular design, which makes possible automatic microscope photometry, image analysis, TV or film microscopy. Other systems covered include the IM, IM 35 and ICM 405 Invertoscopes, all with photographic capabilities.

Reader Service No. 132.

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